

More patent troubles about genes

Two decisions by the British Appeal Court raise questions about the allowable breadth of biotechnology patents, but point to the charges patent-holders should be free to make.

As night follows day and vice versa, so the patenting of genetic information is once again an issue of public policy (see page 487), this time in the British courts. Two US biotechnology companies, Biogen and Chiron, have been challenged in the Appeal Court over the validity of the broad patent protection they have secured from the European Patent Office for the use of defined antigenic peptides in the development of vaccines against hepatitis B and the testing of blood for contamination with hepatitis C (previously 'non-A-non-B') respectively. The Appeal Court decided that Biogen's patent claim is too broad, but Biogen is appealing to the House of Lords, whose judicial committee is the next best thing to a supreme court in Britain. Chiron, on the other hand, has been given an injunction against the British company selling diagnostic kits for hepatitis C pending the full hearing of its appeal.

Reports in the general press that Chiron has been awarded the right to protect a "naturally occurring gene" are an exaggeration; only the full hearing of the Appeal Court can decide that. But both patents raise the question of how broad can be the protection for which an inventor may legitimately apply. Readers of the arcane patent literature will be familiar with the way in which inventors and/or their lawyers multiply their claims for their invention. This is the spirit in which the inventor of a mousetrap will claim that his trap will kill not only mice, but "(2) other rodents; (3) all quadrupeds; (4) all bait-seeking animals", and so on.

These exercises in imagination appear often to be designed to tease the patent-examiners. The question that needs to be decided is how broadly, outside the scope of whatever research may have been carried out, a patent may legitimately be drawn. Another, not raised overtly in last week's court decisions, is that of the reward a patent-holder may legitimately claim from licence-holders.

Nobody dissents from the view that patents are a means by which those who have spent money on research and development are rewarded with a licence of limited term for the exploitation of their inventions. The quid pro quo for the term-monopoly is that the data on which the claims are based, including the patent itself, should be published; that ensures that the technique becomes public property once the patent has expired. Without such a system, the funds spent on research and development by private corporations would be much reduced.

So how broad may a patent legitimately be? The Chiron case is a handy illustration. The first step appears to have

been the identification of an antigenically effective epitope serving as the basis for a reliable blood test. That is the product of meaningful research. On the assumption that the patent application includes (or refers to) data demonstrating the antigenic effectiveness of the particular peptide, it must then be asked what the situation would be if a patent were granted for the use of that epitope alone. Competing companies could set out to find another. Presumably, such a peptide would not be patentable; once the principle had been demonstrated that one epitope could make a blood-test (or a vaccine), it would be considered 'obvious' that a second would function similarly. But then the value of the patent-monopoly would be enormously diminished. In short, patents proved in respect of a single sub-sequence should also extend to other sub-sequences whose use in the same application is obvious. But an epitope proved effective in a blood-test does not, on that evidence alone, make a vaccine.

The Biogen case is more difficult. Again there appears to be an epitope whose effectiveness as a vaccine has been demonstrated. These days, there should be no cavilling about the means by which the peptide is produced in bulk; Biogen could indeed consider itself cheated of protection if others could make and sell the same material by the use of a vector other than *E. coli*. But it is far from obvious (in the patent lawyers' sense) whether any other epitope will serve as well. The effectiveness of various epitopes in stimulating an immune response is notoriously variable. That is very much a matter of judgement, on which the House of Lords will have to pronounce.

The royalty charged for the use of patented materials is also a matter of judgement, on which the patent-holder should not be (and is not) the sole arbiter. Patent-holders are naturally inclined to expect that their income from patent royalties can be equated with some sub-multiple of their total spending on research, but much of their research will have been misguided. It is understandable that British blood-products organizations should be offended that Chiron plans to charge much more for its blood-testing kits than the unpatented products now on the market. It does not follow that Chiron's licensees' proposed charges are onerous nor, equally, that they are reasonable. What the patents system needs is a means of telling by arbitration whether intended royalty charges are fair. Most governments have the right to compel compulsory licences if a patent-holder fails to make patented products readily available. Excessive royalties should trigger such a finding more often than they do. □



Nervousness on money

Even well-heeled governments are hostages of global markets, and should band together for greater strength.

FIRST, the good news: the US Congress and the Japanese Diet have both ratified the international treaty negotiated at the end of last year that will bring into effect the Uruguay round of the General Agreement on Tariffs and Trade (GATT) and, at the same time, replace GATT by the World Trade Organization. That removes the prospect that the GATT agreement might languish in a legislative limbo, at the mercy of Mr Jesse Helms and his allies in Washington, and that the world would be denied the benefits of the further liberalization of world trade for which the agreement legislates. Indeed, if this agreement had not been delivered, the chances that existing GATT agreements would have begun to unravel would have been substantial.

The other side of that coin is the continuing nervousness of the world's major governments about money. It is a curious situation. In the United States, where it appears that the recent recession has given way to economic growth, every sign that growth may be sustained is the occasion for anxiety. People start worrying that the Federal Reserve will increase interest rates, the bond market takes another dip and the population edges onto tenterhooks. This week's alarm is that the number of people estimated to be out of work has again fallen. Next week it will be something else.

Britain is in worse case. The government's budget for the year beginning next April, read out to the House of Commons last week, hangs on the expected reduction of unemployment benefit that recovery from the recession will bring to reduce the government's annual borrowing, but the budget also relies on a planned increase of value added tax on fuel by 10 per cent to raise roughly 2 per cent of the government's annual spending. Now there is a chance that the 2 per cent will not be forthcoming. A group of the government's own supporters plans to sabotage the pre-planned increase (and may have done so on Tuesday this week). In that case, the government argues, there will have to be a significant increase of interest rates, perhaps by 2 per cent, so as to reassure the financial markets that the government is serious about interest rates. Some of the same sensitivity is evident in Germany and France. Italy, whose budget is still the hostage of its pensioners, could yet take a tumble on the markets.

The reason for governments' sensitivity to the markets is a simple consequence of an old phenomenon: globalization in the money markets. Britain's exit from the European Monetary Union two years ago was the consequence of scepticism among international moneybags about its capacity to hold to the agreed exchange rates. Some of Britain's prosperity in the past year, on the other hand, is a consequence of US purchases of shares in British companies (estimated at an extra £12 billion over the year), which could easily melt away again if US investors became nervous about Britain's prospects. But that is only one reason why the government has to be resolute in battling against inflation.

And exactly the same is true of the United States, where government debt is still largely represented by overseas holdings of government securities.

Life would be simpler if governments' finances were part of a larger pool. Not only would overseas investors be less able to bet against the stability of individual currencies but they might find that they had nowhere else to put their money but in large and thus more durable investment pools than there are at present. In Europe, that is a case for a commonly managed currency, of the kind that the European Union is committed to make a reality at some point in the near future — and out of which Britain has won the right to opt. That now seems a hollow victory, as the delicacy of this year's budget balance shows. □

Public understanding

Politicians have a responsibility to say openly where they stand on the activities of the research enterprise.

As motherhood used to be regarded as a universal virtue, so now is the public understanding of science. The research community is one of the principal supporters of the cause, and for good reason. There are two potential benefits to be won: a more sympathetic and general understanding of the purpose of research, and thus a more generous view of the scale on which it should be supported, and a greater enthusiasm among young people for careers in research.

In the United States, the government supports the cause directly through the National Science Foundation and indirectly through the programmes mounted by the national laboratories for involving schoolteachers and even students in scientific activities. In Britain, the Office of Science and Technology now (to its credit) directly supports (on a very modest scale) the work of well-intentioned voluntary organizations, the British Association among them. Echoes of these activities are found elsewhere.

In the event, the benefits of these arrangements are not easily defined. Entrants to the research professions continue to dwindle in number, while gross misunderstandings of technical issues continue to be issued by pressure groups of various kinds — and often pass unchallenged by the organizations at which they are malevolently directed. The result is that the modest efforts by the research community to win the battle for understanding are usually offset by meretricious efforts in the opposite direction.

The missing element in this argument is the positive engagement of governments in the argument about the benefits that may yet be won from understanding. To be sure, governments are ready enough to defend their spending on research by reference to the wealth that will ultimately be created, but they studiously avoid engagement on matters as various as the dangers of the emergence of new genetic knowledge and the safety of nuclear power plants. Nowhere, now, are there politicians ready to speak up for science in the particular, not just the general. That is why the campaign for understanding languishes. □

House of Lords is asked to rule on breadth of gene patent coverage

London. Britain's House of Lords has been asked to adjudicate on an issue of key importance to the future of the biotechnology industry, namely the legitimate breadth of the patent claims that can be based on a particular genetic invention or 'discovery'.

At issue is the extent to which a company holding patent rights — for example, to part of a viral genome or to a key disease gene — should be allowed to demand licences from other companies wishing to use related genetic information to develop diagnostic or therapeutic techniques.

The appeal to the House of Lords was made on Monday by Biogen Inc., the US biotechnology company, after the Court of Appeal overturned its patent on recombinant DNA techniques for producing a vaccine against hepatitis B, in particular on the grounds that the claims made in the patent were too broad.

The patent described a technique — developed by Ken Murray of the University of Edinburgh — for producing hepatitis-B antigens by inserting DNA from the virus into an *Escherichia coli* vector. But it extended the claim to all similar techniques for producing antigens, whether for core or surface antigens, bacterial or non-bacterial hosts.

The court's ruling has come as a surprise to many patent lawyers, as it seems to conflict with the European Patent Convention. This states that the breadth of a patent cannot be challenged once it has been issued, an

interpretation recently confirmed by the appeals board of the Munich-based European Patent Office (EPO).

But the ruling was warmly welcomed by Medeva, the UK pharmaceutical company that challenged Biogen's patent. It had previously failed to block the application at the EPO, where Biogen obtained a patent on its hepatitis-B vaccine in July 1990.

Medeva is one of several companies seeking to challenge broad patents on the grounds that they give companies monopoly control over large fields of technology. The US

Chiron Corporation, the Californian biotechnology company that discovered the virus in 1987.

The court in London upheld an injunction requested by Chiron to stop Murex from continuing to make and sell hepatitis-C kits (Murex currently supplies around one-third of the British market). Murex lost a challenge to Chiron's patent last year, but wanted to continue producing kits until its appeal against this decision has been heard.

The new ruling has provoked widespread concern in the blood-testing research community, partly because it prevents Murex — which does not hold a licence on Chiron's patent — from carrying out further research on screening techniques.

"It is not in the public interest for a company to be able to claim the whole area of a particular disease just because it has rights to a few sequences specific to the genetic material related to that disease," says Roger Williams, head of the Institute of Liver Studies at Kings College Medical and Dental School in London, and director of the Liver Research Trust.

One concern of such critics is that only companies that license from Chiron — at present, Abbott Laboratories, Ortho and Pasteur — will have access to the UK market for hepatitis-C screening. They also point out that hepatitis-C tests cost three to four times more than other virus tests because of the royalty payments fixed by Chiron, said to be about £1 on a test that costs 50p to make.

Another is that Chiron's rights to the hepatitis-C virus could eventually give it leverage over the key technologies used for blood screening worldwide. These are likely to be based on automated equipment that simultaneously screens for several viruses, including hepatitis C, and Chiron's approval would therefore be required by any producer of such equipment.

"There is a general feeling in the blood transfusion service that, because of the way the technology is moving, the implications of this ruling are more serious than the issues raised by hepatitis-C screening alone," says one scientist who has been closely involved in developing novel screening techniques.

But officials at Chiron and its licensees deny that they are seeking to monopolize either research into techniques for hepatitis-C screening, or the global blood-screening industry — the market for test kits is around £200 million (US\$314 million) a year. "Chiron has poured a lot of money into research in this area, which it is entitled to recoup," says John Menzies, sales director

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Blood testing: who will control the technology?

diagnostics company Murex International, will also be keen for any clarification of the issue that the House of Lords might provide. Its UK subsidiary, Murex Diagnostics, last week lost a further round in its worldwide challenge to the validity of a patent on a blood-screening test for hepatitis C, held by

Russians may lobby for science aid

Washington. A group of Russian scientists is discussing ways of paying for a Washington lobbyist to boost efforts to persuade the Clinton administration to contribute money to the International Science Foundation (ISF), the research-funding agency set up by Hungarian-born financier George Soros.

The decision to employ a lobbyist was taken two weeks ago at a meeting of ISF grantees in Moscow. "If we do not receive more money, then funding for Russian scientists through the ISF will finish at the end of next year," says Vladimir Skulachev of Moscow University, the chairman of the ISF's Russian advisory committee.

Soros initially established the ISF with a gift of \$100 million, and later promised extra funding if this could be matched by other donors. In response, the Russian government has already promised to provide a further \$12.5 million, which Soros has agreed to match.

But despite lengthy negotiations with the State Department in Washington (including numerous requests from the US scientific

community), the Clinton administration has so far refused to come up with any funding for the foundation.

The Russian scientists, who have pledged to provide the money out of their own pockets for a Washington lobbyist — as a non-profit organization, the ISF is unable to engage in lobbying activities on its own behalf — are hoping that the foundation's future will be raised at a meeting in Moscow next week between US vice-president Al Gore and Russian Prime Minister Viktor Chernomyrdin.

The Russian science minister, Boris Saltykov, has already been approached to try to place the future of the ISF on the meeting's agenda. But, with many other issues already scheduled for discussion — including US/Russian cooperation in space research — the prospects for this remain uncertain. As Roald Sagdeev of the University of Maryland's East-West Science and Technology Center, puts it: "The biggest problem for Russian science is not so much survival in the post-Soviet epoch, but survival in the post-Soros epoch." □

► of Ortho Diagnostic Systems.

Mr Justice Aldous, the High Court judge responsible for the judgement, agreed. "Chiron's patent monopoly will in the short term deter some companies from carrying out research and development," he wrote. "But that is inherent in the patent system."

Aldous said he accepted that a patent holder's rights to restrict competition and put up prices was "contrary to the public interest". But he added that this was "the price that has been accepted to secure the advantages" that the patent system offers to inventors.

Indeed, the ruling against Murex — upholding Chiron's claim on future technologies for detecting hepatitis C — conforms with recent decisions by the EPO to grant broad patents, for example on thermostable polymerases used in the polymerase chain reaction. But such rulings, as well as similar decisions in the field of agricultural genetics (see right) are coming under increasing fire both from the scientific community and from small companies.

There is a growing sentiment in the research community that broad patent claims on basic genetic discoveries may be creating an unacceptable hurdle to researchers seeking corporate sponsorship for their research, because companies may be unwilling to support research whose potential products are owned in advance by others.

Murex and other companies contesting broad patents on the grounds that they provide an unfair monopoly are turning to 'public interest' arguments to support their case. "The patent system has perhaps gone too far [in allowing broad patents]", says Peter Silverstone, director of business development at Murex Diagnostics. "Patents should protect something that is specific."

Chiron is quick to dispute charges that broad patents discourage research, as are others in the biotechnology industry, such as Genentech, Amgen, Eli Lilly, Hoffmann La-Roche and SmithKline Beecham. This is hardly surprising, given that their commercial success increasingly depends on being the first to patent key elements of know-how, ranging from individual gene sequences to basic gene-sequencing technology, and subsequently exploit the market position that such patents allow.

If the House of Lords does review the Appeal Court's rejection of the Biogen patent, this will come too late to influence Murex's appeal, due to be heard next year, as the Lords hearing is unlikely to take place until 1996. But it would set an important precedent for the whole European biotechnology industry.

Furthermore, the House of Lords' views on gene patenting, which is already being considered separately by a select committee, are also likely to be closely examined in US circles, given for example the current debate there (see right) over 'reasonable pricing' clauses in joint industry/federal research projects.

David Dickson

Soy-bean patent comes under fire as threat to research

San Francisco. An international coalition of public-interest groups and the multinational company Monsanto have filed separate challenges to a broad European patent granted to an American biotechnology company on all genetic manipulation of soy-bean plants.

Both appeals have highlighted a mixture of concerns being expressed by activist groups, government agencies, researchers and sometimes other companies in the industry about the threat that broad patents may pose to research, the dangers of a monopoly over major food crops and the potential for loss of plant diversity.

Monsanto claims that the scientific data in the soy-bean application supported a patent of far narrower scope. Jim Altemus, spokesman for the chemical company, which is based in St Louis, Missouri, says the broad coverage of the patent may discourage others from entering the soy-bean area and carrying out original research not contained in the patent. "It's just too broad in coverage," says Altemus. "We're not objecting to patents *per se*."

The patent, issued by the European Patent Office on 2 March, means that any company or researcher interested in genetically engineering soy beans must arrange licensing from Agracetus Inc., a subsidiary of W. R. Grace & Co. based in Middleton, Wisconsin.

Agracetus, which uses 'gene-gun' technology to move new genes into soy beans, won the broad claim on the grounds that its scientists were the first to show that the

species could be genetically engineered by any method.

But the Rural Advancement Foundation International (RAFI), an Ottawa-based biotechnology watch-dog group that led the activist coalition's complaint, argues that the invention is neither novel nor 'non-obvious', as it is based on previous reported discoveries.

RAFI also says the patent is morally wrong because it would grant a single company a 17-year monopoly on genetic research on one of the world's most important food crops. Its challenge is intended to send a strong signal to the biotechnology industry — and to patent officials — that patenting major food crops is unacceptable, said Hope Shand, research director for RAFI-USA in North Carolina. She said the patent system was not designed for genetically engineered plants and other living things and is ill-equipped to handle them.

But Russell Smestad, vice president of finance and commercial development for Agracetus, argues that strong intellectual property rights have historically stimulated more active research and investment.

Agracetus has filed for a similar broad-based patent on soy-bean manipulation in the United States and Canada. It already holds a US patent for all transgenic cotton products, which is being re-examined by the US Patent Office following a challenge by the US Department of Agriculture and an anonymous party.

Sally Lehrman

NIH 'should rethink pricing clause'

Washington. The US National Institutes of Health (NIH) were urged last week by various members of their advisory councils to drop an unpopular clause that forms part of cooperative research agreements between industry and NIH intramural scientists.

The controversial clause allows the NIH to set "reasonable prices" for products developed jointly with industry. It was introduced following congressional and public anger over the cost of the anti-AIDS drug AZT, which was partly developed in NIH laboratories.

Edmund Tramont, a member of the advisory council to the National Institute of Allergy and Infectious Diseases, last week told a meeting of the advisory committee to Harold Varmus, the director of NIH, that the Public Health Service is now sophisticated enough to guard against the unreasonable use of technology developed with public money. "I don't think the AZT case will arise again," he said.

Both David Guyton, an adviser to the National Eye Institute, and Timothy Wright, representing the National Institute for Dental Research, agreed. "The bottom line is that reasonable pricing clauses restrict cooperative research agreements," said Wright.

The NIH has spent much of the past year trying to decide what to do about such clauses. Many companies say they will not enter research agreements containing the clause because it discourages investment by venture capitalists.

Earlier this year, two *ad hoc* panels of NIH staff, extramural scientists and industrialists each agreed that something needs to be done about the clause as it now stands. But it remains unclear what the NIH, under pressure to revise the current clause but also to respect the need to protect public investment in research, will eventually recommend to the Public Health Service of the Department of Health and Human Services.

H. G.

Academy report backs 'science for all' plan

Washington. A radical overhaul of school science in the United States, with a far greater emphasis on 'understanding' and less on 'facts', is proposed in a draft set of national science education standards released last week by the National Research Council, the operating arm of the National Academy of Sciences (NAS).

The ambitious standards — which the authors admit represent a long-term vision rather than a realistic short-term prospect — call for all children at elementary, junior high and high school in the United States to reach a comprehensive and demanding level of scientific literacy.

They lay strong emphasis on the need for schoolchildren to understand concepts from an early age, rather than just learning facts and equations. Such concepts range from the nature of scientific enquiry to the relationship between science and health.

"We want to de-emphasize words and replace them with understanding," said Bruce Alberts, president of the academy and a long-standing advocate of improved science teaching, in unveiling the draft. He attacked the traditional teaching of science in US schools in forthright terms: "we fail to convey what science is, and we kill off the curiosity of the kids".

The draft, prepared at a cost of \$6.5 million, mostly paid for by the Department of Education and other US government agencies, says little about where resources would come from to pay for the expansion of science education implied in the proposed standards. But Alberts says that the commitment of parents, teachers and local school districts will be more important than extra money in implementing the standards.

The committee that drew up the document over the past two years was chaired by Richard Klausner, a senior cell biologist at the National Institutes of Health. He stresses that the standards are not an attempt by the federal government to impose uniformity on science education. "These are not federal standards, nor are they government standards," he says. "They are grass-roots standards, for the people who make decisions [on education] at the local level."

The document calls for greater participation by children in the learning process, and concedes that the teaching of very basic principles, such as the nature of an organism or the fact that all objects have properties, will involve a trade-off against the number of scientific 'facts' that children can learn. "If students are to understand the 'big ideas' of science, they will have less vocabulary, fewer topics, and reduced memorization of information," it says.

The content standards in the document call for a broad range of key principles to be taught to all pupils of all ages. In Earth and space science, for example — one of eight

domains to be taught to every student — pupils in grades K to 4 (aged 5–9) are expected to learn to understand "properties of Earth materials" and "objects in the sky".

At grades 5 to 8 (aged 10–13) they should learn the "structure of the Earth system", "Earth's history", and "Earth in the Solar System". From grade 9 to 12 (aged 14–17), every teenager would learn more sophisticated concepts: energy in the Earth system, geochemical cycles, and the origin and evolution of the Earth and the Universe.



Klausner: 'no attempt to impose uniformity'

Other domains include the history of science, and science and society, as well as the traditional fields of physical and life sciences.

The document states unequivocally that none of these content elements are optional, and that each is necessary for a scientifically literate US population. Challenged on the realism of such a goal, Klausner said that his panel "had to set a vision which is far from where we are now", and that the levels of scientific literacy laid out in the standards document were "not unrealistic".

The authors are optimistic that the science standards can avoid the kind of political controversy that has engulfed recent proposals for history teaching standards in the United States. For example, they steer cautiously around such awkward subjects as

creationism, saying that it should be addressed if raised by students.

Klausner denies that there is a 'back to basics' movement in US schools, and says that the progressive ideas in the document have widespread support from both parents and the local school districts that run the US school system. But Alberts concedes that some parents remain skeptical. "I'm convinced that if people really understood what we were talking about they would support it," he said. "But we have a big communications problem."

Around 30,000 copies of the standards draft will be circulated as part of a vast consultation process. Like other standards being proposed for schools, the science standards are the culmination of a process that began in earnest at the National Conference of State Governors in 1989.

The process is driven by a widespread fear that US school education is falling short of European standards and is well behind standards in the Far East. Many academics report that US college entrants are two years behind their overseas peers at the age of 18, but that the well-organized and wealthy US university system makes up the ground lost in the schools.

But the proposed science standards focus less on potential college entrants than on achieving basic scientific literacy in the rest of the population, including those from disadvantaged backgrounds. Despite recent initiatives from the National Science Foundation and others (see *Nature* 369, 87; 1994), the state of school administration in many US cities will make that objective extremely difficult to meet.

Colin Macilwain

OTA under threat from US Senate

Washington. Robert Walker, a conservative Republican from Pennsylvania, is to chair the House of Representatives' committee that oversees most US federal science programmes when the new Congress meets in January. Walker, who lost a bid for his party's Whip position on Monday, will take over the Science, Space and Technology Committee formerly chaired by Democrat George Brown of California.

That committee also will get a new name — Technology and Competitiveness — intended to reflect the Republican majority's concern for economic issues. As a result of a re-shuffling of committee assignments, it will also assume greater responsibility for research programmes at the Department of Energy's national laboratories.

But the committee will maintain its oversight of both the National Aeronautics and Space Administration and the National Science Foundation, as well as civilian research at several smaller agencies. The Na-

tional Institutes of Health will stay under the jurisdiction of the Commerce (formerly Energy and Commerce) Committee.

Although the most sweeping Congressional changes are likely to be in the House, Republicans in the Senate are also stirring things up as well. Last week, the Senate Republican Conference recommended that the Office of Technology Assessment (OTA), which provides independent advice to Congress on science and technology matters, should be abolished as a money-saving measure.

The attack, spearheaded by Senator Pete Domenici of New Mexico, caught OTA and its supporters by surprise. Although they believe OTA ultimately will be rescued in the appropriations process, the agency clearly will be on the defensive during the next session. "In this atmosphere, it's prove why you shouldn't be cut' rather than vice versa," says one Congressional staff member.

Tony Reichhardt

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Japan's opposition falls into line on plutonium policy

Tokyo. The last vestige of domestic political opposition to Japan's policy of using recycled plutonium in nuclear power melted away last week, when the Social Democratic Party (SDP) backed down on its longstanding criticisms.

The SDP was the leading opposition party during 36 years of Liberal Democratic Party (LDP) rule that ended last year, and since then has been a member of the ruling coalition government. At a meeting to set budget allocations for the Science and Technology Agency (STA) in 1995, the SDP, which is headed by the prime minister Tomiichi Murayama, abandoned its role as champion of the anti-nuclear movement.

Instead, the party accepted the LDP policy calling for more money to be spent on explaining the need for plutonium to the public. Among the new initiatives passed by the meeting is a plan to release information on Japan's plutonium use on the worldwide Internet computer network.

Japan's plans to import and subsequently to produce large amounts of plutonium has raised objections from around the world, many triggered by the start of imports of reprocessed plutonium from Europe by ship last year. Furthermore, Japan has found itself in the politically awkward position of having to criticize North Korea's use of plutonium at a time when it is planning rapidly to expand its own supplies of the material.

At first, SDP members of the coalition government insisted that the plutonium issue be reviewed and its use capped at current levels "in keeping with the sentiment of the international community". The SDP also proposed that the electric power industry should be prohibited from using plutonium where it was not profitable.

But Japan's programme to develop com-

mercial fast-breeder reactors able to use the plutonium as fuel has been repeatedly delayed (see *Nature* **369**, 569; 1993). To cope with the growing supplies of plutonium (expected to reach about 70 tonnes by 2010) that cannot be stockpiled without violating the Nuclear Non-Proliferation Treaty, Japan's nuclear-power industry plans to mix it with ordinary uranium fuel and burn it in conventional reactors.

This is a very uneconomical way to use plutonium. But the SDP, which has seen some of its key policies compromised during the coalition administration, had its opposition to the current plutonium policy quickly squashed by members of the LDP at the meeting.

Instead, ¥142.1 billion (US\$1.42 million) has been allocated to the STA for the "steady development of the recycling of nuclear fuel" in 1995, two per cent more than in 1994. Included in the allocation is ¥110 million for two "public information rooms", and another ¥20 million for release of information on Internet.

This has already begun. The week before the budget meeting, the Atomic Energy Commission published for the first time a detailed breakdown of Japan's inventories of plutonium in its annual white paper (see table below). This showed that, as of December 1993, Japan had about 10 tonnes of plutonium, with about 4.7 tonnes stored in Japan, and the rest in reprocessing facilities in Britain and France.

But although the promoters of plutonium policy seem to have won the day, their critics say that the worldwide release of such information will only lead to greater criticism of Japan's policy, which is out of line with a worldwide trend away from the use of plutonium and fast-breeder reactors.

David Swinbanks

Space scientists are charged in Indian spy-ring scandal

New Delhi. Security at India's space installations has been strengthened following the arrest of six members of an alleged spy-ring, including two senior scientists of the Indian Space Research Organisation (ISRO). The scientists are said to have sold space secrets for sex and money.

Those being held by the Kerala State police include the Indian representative of Glavkosmos, the Russian space agency, and a business associate. Together with the ISRO rocket experts, the two have been charged with passing national secrets to two women of Maldivian nationality. The women are also in judicial custody.

The scandal has shocked ISRO. One of the scientists arrested is deputy director of the liquid propulsion systems centre (LPSC) at Valiamala near Trivandrum, close to India's southern tip. The second is chief of the rocket fabrication division at the same centre.

About 20 other employees holding key positions at the centre are being kept under surveillance or house arrest pending formal charges. Last week, the Central Bureau of Investigation (CBI), India's main security agency, took over the case.

As well as constructing control systems and apogee motors for spacecraft, LPSC makes liquid engines, according to a French design, for ISRO's polar satellite launch vehicle. These engines are identical to an early version of the Viking engines that power the first stage of Europe's Ariane rocket.

The centre is also responsible for the design and construction of the cryogenic stage for India's geostationary launch vehicle. The involvement of the Glavkosmos agent has fuelled speculation that the targets of the alleged spies were the cryogenic engines that Glavkosmos is supposed to deliver to ISRO beginning next year (see *Nature* **368**, 577; 1994).

ISRO says the scandal will not upset its space programmes. Nor will it harm national security, as ISRO does not handle military projects. But officials within the organization acknowledge that the leaked information could help saboteurs.

Although the spy network is alleged to have been operating for at least four years, the CBI is taking no chances. It is now investigating whether there may also be spies at ISRO's other installations, such as Vikram Sarabhai Space Centre in Trivandrum, which designed the first stage of India's ballistic missile 'Agni'.

Indian security agencies believe the spy network was run by a third country, using the Maldivians as either couriers or bait. Pakistani authorities have vigorously denied suggestions that they may have been involved.

K. S. Jayaraman

Separated plutonium at nuclear facilities within Japan.

	PNC Tokai Plant	PNC Tokai Works	Fugen	Joyo	Monju	R&D
Plutonium in nitrate form (plutonium nitrate in process area, storage area or conversion area)	288					
Plutonium in oxide form (plutonium oxide stored in containers at storage areas)	38	2,339				
Plutonium oxide in fabrication process or under utilization such as analytical testing		790				
Plutonium in fresh MOX fuel element (plutonium contained in MOX fuel assemblies at storage area)		140				
Plutonium in unirradiated MOX fuel assemblies held in fresh fuel storage areas at reactor facilities or fresh plutonium used for R&D purposes			15	637	12	425
TOTAL	326	3,269	15	637	12	425

Prospects grow brighter for global biosafety agreement

Nassau, Bahamas. Representatives of industrialized countries last week indicated that they may be ready to compromise over calls by developing countries for a global biosafety protocol. Signs of a possible compromise emerged during the first Conference of the Parties (COP) to the United Nations (UN) Convention on Biological Diversity agreed in Rio de Janeiro in 1992. (The meeting ends on Friday 9 December.)

The need for a biosafety protocol has been advocated throughout negotiations on the convention by delegates representing China and the so-called Group of 77 (G77), comprising some 132 developing countries. But the proposal has given rise to a vigorous debate between North and South.

According to A. H. Zakri, professor of genetics and plant breeding at the National University of Malaysia in Kuala Lumpur, and a Malaysian delegate to the COP, a global biosafety protocol would be made up of a uniform set of rules governing the environmental release of genetically modified organisms. Zakri points out that although Northern countries already have stringent national guidelines, most developing countries do not yet have the expertise to design such laws. One of the aims of the protocol, he says, would be to ensure that "experiments which are deemed not suitable in the developed countries would not be undertaken in developing countries".

Half-way through the two-week conference, Northern delegations appeared to be moderating their previous positions on this issue. Toshiki Kanamori, director of the Global Issues Division in Japan's Ministry of Foreign Affairs, said "we do not have any objection to initiating the process to consider the need for and modalities of a [global biosafety] protocol".

Echoing this position, Rafe Pomerance, deputy assistant secretary of state for environment and development and head of the US delegation, suggested that such "key international organizations as the UN Industrial Development Organization [UNIDO] and the World Health Organization [WHO] might work to inform the COP on the risks of biotechnology and the release of genetically modified organisms". (As the Senate has not yet ratified the biodiversity convention, the US delegation is attending the Nassau meeting as an observer, and is unable to vote on conference resolutions).

Several delegates, while admitting that the issue of biosafety is a valid concern, pointed out that the introduction of alien species into native ecosystems presents perhaps a greater threat to global biodiversity. Some expressed regret that this issue was

not on the agenda of the first COP.

In other areas, the COP was becoming mired in predictable North-South splits. For example, despite widespread consensus that the \$2-billion Global Environment Facility (GEF) administered by the World Bank, the United Nations Development Programme (UNDP), and the United Nations Environment Programme (UNEP) is likely to be the interim funding mechanism to serve the goals of the convention, dissatisfaction with the governance of the GEF was expressed by the G77 and China.

Southern nations are advocating the creation of a new biodiversity fund under the



direct authority of the COP, even though in the present GEF arrangement virtually the same group of nations is represented on the GEF council, with power to approve new environmental projects.

There has been further controversy about Article 18 of the convention. This calls for the establishment of a clearing-house mechanism to facilitate technical and scientific cooperation among parties to achieve the convention's goals of biodiversity conservation, the sustainable use of its components and the equitable sharing of benefits arising from the use of genetic resources.

A proposal to provide global brokering services through this clearing-house mechanism in order to promote equitable trading of genetic resources was put forward by the Swedish delegation, and supported by the G77 and China.

Supporters argue that such a service might facilitate access to technology, as called for in the convention, but on a voluntary basis, as insisted on by Northern governments. But the proposal still provoked objections from various Northern countries. These would prefer the function of such a clearing-house mechanism to be limited to providing scientific information. **Daniel Putterman**

UK promises level funding for science budget

London. The British government announced last week that the science budget for 1995-96 will be £1.282 billion (US\$2.01 billion) an increase of £40.2 million on this year. But the award will not provide any growth in real terms, as the increase coincides with the anticipated rate of inflation.

David Hunt, the cabinet minister responsible for science, described the decision, announced as part of the government's general budget plans, as a "good settlement". Furthermore, he said that because of a lower than expected inflation level this year's science budget had grown by 2 per cent in real terms, equivalent to about £31 million extra.

Robert Hughes, the junior science minister, said that "science has come out again as one of the top priorities". He added that, unlike some of his cabinet colleagues, Hunt had "successfully defended" the science budget by persuading the chancellor, Kenneth Clarke, not to claw back this unexpected bonus.

The new budget has been given a cautious welcome by professional scientific bodies. Sir Michael Atiyah, president of the Royal Society, said he was "pleased" to see that the government "has continued its support for science", and that the settlement "reflects the importance the government attaches to science, technology and engineering in the United Kingdom".

But the pressure group Save British Science (SBS) was less impressed, saying that the sum amounted to "a fudged promise" — the prime minister, John Major, had told the Parliamentary and Scientific Committee in February that "we expect spending on the science base to rise in real terms next year, and science will remain a high priority in future".

The group has told the government that in its view a "rise in real terms" should mean at least a 2.5 per cent increase in the science budget for the next financial year over and above inflation. "This is not the response we expect from a serious prime ministerial statement," said John Mulvey, secretary of SBS.

John Battle, spokesman for science and technology for the opposition Labour party, also challenged the figures. He is concerned that much of the science budget, distributed to the five main research councils through the Office of Science and Technology (OST), will be used to pay for redundancies. "The government is determined to make staff cuts at the research councils' headquarters [at Swindon in Wiltshire] but the OST needs Treasury money to fund redundancies. If their bid for Treasury money has been successful, millions will be wiped off research and teaching budgets to pay for job losses," claims Battle. **Maggie Verrall**

Cancer agency denies mismanagement

Paris. Allegations of serious anomalies in the running of France's largest medical charity, L'Association pour la recherche sur le Cancer (ARC), have reopened a debate about the role of medical charities in the financing of research.

The allegations are contained in a letter written in 1991 by Michel Lucas, director of the Inspection Générale des Affaires Sociales (IGAS), to Claude Levin, then minister of social affairs and solidarity, outlining the preliminary findings of an investigation of ARC in 1990.

The letter, which has now been leaked to the press, has no official status, as an administrative tribunal 'annulled' the investigation two months after it had begun. The court upheld a complaint by ARC's president, Jacques Crozemarie, that IGAS — which is responsible for investigating public authorities — had no right to investigate private associations.

The allegations include a claim that ARC spent 65.5 per cent of its FF430-million (US\$80-million) income in 1989 on "running costs". ARC's income in 1993, FF581.2 million, is around five times the combined spending (excluding salaries) on cancer research by the Centre National de la Recherche Scientifique (CNRS) and the biomedical research organization, INSERM, over the same period.

Lucas's letter also claims that most of these running costs were paid exclusively to three public relations companies, that shared the same management. As a result, it concludes, much of the money collected by ARC was used to "finance the development of external commercial companies".

Lucas describes the advertising campaigns used by ARC to encourage donations as "contestable" (the last issue of ARC's magazine *Fondamental* provides potential donors with a ready-made last will and testament). And his letter also accuses ARC of extravagance, for example in inviting 36 French journalists for a week in China for a three-day 'symposium'.

ARC's board of directors is criticized for failing to exercise proper control over ARC's activities. Moreover, although ARC distributes research funds through scientific peer-review committees, the letter alleges that many board members were "privileged" recipients of grants, and that the board failed to control the use of an "emergency" research fund — around FF20 million — spent at the "discretion" of Crozemarie.

Crozemarie denies the allegations, claiming that IGAS wanted to "destroy" the association "for political reasons". The investigatory body overestimated ARC's running costs, he argues, by including activities in "prevention and information".

Dominique Bellet, director of the immunochemistry laboratory at the Institut Gustave Roussy at Villejuif near Paris and a member of the ARC board, also denies the allegations, pointing out that the board also includes representatives of CNRS, INSERM and government ministries. ARC paid Bellet FF16 million to set up his laboratory, and pays up to 40 per cent of its running costs.

Reaction in the research community to the letter has been mixed. One widely held view is whatever the validity of the allegations, ARC has inevitably come under suspicion because it has not been sufficiently

open about how it spends its money.

Another concern is that the adverse publicity could provoke a public backlash against medical charities, upon whom many public laboratories depend heavily for support. This concern is shared by Philippe Douste-Blazy, the junior health minister, who last week called for "absolute transparency" in the management of medical charities.

The first test of the public's reaction came last week when the French Muscular Dystrophy Association (AFM) held its annual Téléthon to raise funds for genetics research. Television personalities, apparently concerned about the implications of the allegations against ARC, assured the public of AFM's accountability, and the pledges received broke last year's record.

Many scientists say they are reserving judgement until the findings of a six-month investigation of ARC by the national audit commission are released later this month. Crozemarie says he is convinced the report will exonerate ARC.

Declan Butler

Officials concede US fusion is 'stuck'

Washington. The US fusion energy research programme has no money to meet its goals, government officials told a group of advisers in an unusually blunt assessment of the \$360 million-a-year programme's prospects.

"We're stuck, frankly," Anne Davies, associate director for fusion energy at the Department of Energy, told the first meeting of a re-modelled Fusion Energy Advisory Committee (FEAC) last week. "We are faced with restructuring the whole programme. We haven't done it yet, and I don't know what it's going to look like."

Over the next five years, the United States wants to fund the Tokamak Physics Experiment (TPX) at the Princeton Particle Physics Laboratory in New Jersey, to participate fully in the International Thermal Experimental Reactor (ITER) with Russia, Europe and Japan, and to maintain a \$220-million basic research programme.

But in presentations to FEAC, Davies and Charles Curtis, the under-secretary for energy, said that the programme could expect no more than continued funding at the existing level. "The implication is that when TPX peaks, it will force down the base programme to \$112 million," said Curtis.

Congress has yet to approve construction of TPX or ITER. "I think it is a very serious question whether Congress will make either investment," Curtis said. He also suggested that earlier Republican proposals to halve the fusion budget were being taken very seriously in the department.

Colin Macilwain

AIDS summit promises left hanging

Paris. Leading politicians from 42 countries, including a handful of heads of state, met in Paris last week to make impassioned speeches on the need for 'solidarity' in the fight against AIDS. They then went home, leaving the question of how much talk will be translated into action.

The leaders signed a declaration committing them to provide resources for seven 'concrete' programmes: to reduce HIV infection of children, reduce the vulnerability of women, improve blood safety, support community associations, reinforce healthcare facilities for AIDS patients, strengthen human rights, and promote 'global collaboration' on research.

Activist groups protested outside the meeting, and called for an end to discriminatory laws. Donna Shalala, the US secretary of state for health, said it could take one to two years before the United States'

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

ban on seropositives entering the country is lifted. Russia, whose parliament last month approved compulsory HIV screening of foreigners, was reported as saying that it would review this decision.

Meanwhile, Paris marked AIDS day by placing an illuminated red ribbon (above) 40 metres high on the Eiffel Tower. All entrance fees to the tower on 1 and 2 December went to AIDS associations.

D. B.

Transport policy revisited

SIR — It is surprising that your leading article about the Royal Commission's report on transport and the environment, "The motor-car as black sheep" (*Nature* 372, 115–116; 1994), concerns itself with the politics of transport to the virtual exclusion of scientific and technological issues, including sustainability. It is even more surprising that you show little awareness of what the report actually says.

You accuse the Royal Commission both of ignoring the attachment of British voters to the car and of succumbing to a "populist animus" against it. In fact the report recognizes, and documents, the advantages of car use, and the extent to which this is a necessity for many people, especially in rural areas. It also sets out clearly and objectively the serious consequences for the environment, and for the quality of life in Britain, if recent overall trends continue unchecked for the next 25 years and beyond.

It should be emphasized that the target proposed for carbon dioxide emissions from transport is to reduce them at the same rate as emissions from other sectors. We show in appendix D how that target can be achieved by measures that are not at all 'draconian'. For fuel duty we recommend an increase of 9 per cent a year in real terms; the government is already committed to an annual increase of at least 5 per cent. A major reason for our recommendation was to give strong encouragement to economy in the use of fuel. For cars that achieve our proposed target for increased fuel-efficiency, fuel costs would be broadly the same as at present.

You ignore the scope for increased fuel-efficiency in conventional vehicles. You also believe there will be major increases in fuel prices in any case as a result of market forces, although we found no evidence that this is likely over the next 25 years. We considered direct regulation (as in the United States or current German proposals), as a possible alternative means of ensuring that improvements in fuel-efficiency actually take place, but concluded that an economic instrument, in the form of increased fuel duty, is much to be preferred.

Although your article concentrates on the issue of greenhouse gases, this is only one of the eight objectives the Royal Commission proposed for a sustainable transport policy. The recommendations about the national trunk road programme follow from some of the other objectives, not (as you claim) from the targets for carbon dioxide emissions.

In particular, the Royal Commission places great importance on the environmental costs imposed by new infrastructure, such as loss or disruption of habitats,

visual intrusion and severance of communities. We did not find it possible, however, to estimate a money value for these costs. The estimates of environmental costs that you quote from the report therefore relate only to quantified costs, and we make it clear (7.17) that total environmental costs would be considerably higher.

Even if all external costs could be quantified, chapter 7 of the report explains why attempting to match costs and benefits at the margin is much more problematic than you imagine.

You recognize the dilemmas that must be resolved in order to find a sustainable transport policy. Given that these dilemmas affect every member of society, our report showed in detail why a fundamentally different approach to transport policy is now required, which will achieve an appropriate balance between the car and other modes of transport.

John Houghton
(Chairman)
*Royal Commission
on Environmental Pollution,
Church House,
Great Smith Street,
London SW1P 3BL, UK*

A creative science

SIR — Your 125th birthday celebrations gave us the chance to look both back and forwards. As director of the institution where you were kind enough to hold your birthday party (and where you also held your centenary symposium) I hope you will not think me churlish if I draw attention to a remarkable omission from the span of topics celebrated.

The capacity audience at the Royal Institution heard authoritative persuasive accounts of our search for ultimate regularities in matter at the microscopic and macroscopic limits, and explanations of the extraordinarily diverse structures found on length scales in between. From particle physics to cosmology, via biology, we were treated to a breathtaking panorama of the present state of human understanding. Nevertheless, all the science on display was occupied with explaining what *is* — with those structures and processes that mould the world we see. Yet there is one science, wholly missing from your programme, that occupies itself not just with observing the world, but with changing it.

Chemical synthesis rearranges the atoms in the cosmos the Good Lord gave us, producing new architectures of matter that simply were not there before. And having done so, it gives us properties the Universe had never seen. Until 1988, it

must be counted extremely doubtful whether the elements Y, Ba, Cu and O had ever come into conjunction since the Big Bang, still less in the right atomic ratio to produce a substance with no electrical resistance at 90K. Those marvellous confections of C, H, N and O (with a smattering of other elements) that make up the biological world have also been creatively interfered with by chemists for more than a century, but with ever-growing vigour since you held your centenary party: we call them pharmaceuticals. Not only do they make us better, they make the economy better, too.

So let us celebrate the creative science that puts atoms together in new ways, synthetic chemistry.

Peter Day
*Royal Institution,
21 Albemarle Street,
London W1X 4BS, UK*

Black and blue

SIR — During the past 20 years it has been the fashion at scientific meetings to present results with slides using white text on a blue background, which I will call blue slides. My assertion is that blue slides reduce readability and comprehension by at least about 20 per cent. It is best to use black text on a white background, or, if one absolutely must use colour, to use dark colours for the text on a faint coloured background such as faint blue or faint yellow.

Two examples illustrate the case. First, the renowned advertising expert, David Ogilvy, claimed that by changing an advertisement from white text on black background to black text on white, a charity organization doubled the income from an advertisement¹.

Second, IBM has found that we read computer screens, that is, white text on dark background, 20–30 per cent more slowly than the same text on paper, black text on white. This difference vanished when a high-resolution screen with black text on white background was compared with text on paper². Apple has utilized this fact for a long time with the Macintosh, where normal use is black text on white background. Most Windows programs also use black text on white background.

There is only one group of scientists who might find it an advantage to use white text on black background, and that is X-ray physicians.

Steinar Øvrebo
*National Institute of
Occupational Health,
Pb 8149 Dep.,
N-0033 Oslo,
Norway*

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Violence and UN population data

Peter Uvin

Burundi and Rwanda have been hit before by atrocious cases of ethnic violence. Why are there discrepancies between these occurrences and the United Nations population data?

In Rwanda, the end of colonialization in 1959 coincided with a Hutu takeover and the abolition of the monarchy. Following this, the Tutsi were subject to years of revenge: arbitrary treatment, torture, confiscation of possessions and killings. In March and April 1962, more than 2,000 were killed and in December 1963, at least 10,000 more were massacred. About 15,000–20,000 people were killed between 1963 and 1964. During this time, between 140,000 and 250,000 Tutsi fled the country — 40 to 70% of the surviving Tutsi population¹. Almost none returned. The Rwanda Patriotic Front, which invaded Rwanda in 1990, is composed mainly of these refugees and their descendants.

In Burundi, the outbreaks of violence have been disturbingly regular. In 1965, after a failed *coup d'Etat* by Hutu officers, popular unrest broke out in which about 500 Tutsi and between 2,500 and 5,000 Hutu died. In 1972, similar events took place, but on a much larger scale. The fully Tutsi-controlled army, called in to end a Hutu rebellion in a southern province, went on a 2-month rampage. According to most observers, 2,000 Tutsi and 100,000–150,000 Hutu were killed, and 150,000 people fled^{2,3}, some of whom returned some years later. In 1988, there was again violence in two villages followed by army intervention: 3,000 Tutsi and between 3,000 and 20,000 Hutu were killed and there were tens of thousands of refugees. In 1991 and 1992, hundreds died while thousands more fled the country⁴.

These occurrences of violence are easily the worst cases of the systematic killing of minorities in the past 50 years. In the light of these awful facts (and, among the various available estimates, I have always chosen the lowest), it is interesting to look at the population data provided by the United Nations system. Given population sizes of only 2.5–4 million for both Burundi and Rwanda at the time of the main events, there should be clear indications of the impact of these violent spasms in the official statistics. The UN data for Burundi and Rwanda on yearly population increases (the absolute number of people added every year to the previous year's population) provide a more subtle indicator than overall population size or population growth rate (Fig. 1). If correct they should very clearly show a decreased or reversed population increase for the years in question.

The United Nations regularly updates its population data, based on new cen-

suses and other available data; the updates are always retrospective by some years. The time series currently in use covers the period 1961–92. This creates a particular problem for Rwanda, as the violence that occurred in 1959–64 cuts through the beginning of this time series. For that reason, I present two different time series: one for 1950–75, made by the United Nations in 1979 (the latest series going back to 1950)⁵, and the other for 1961–92, made by the United Nations in 1993 (ref. 6). This cut-off problem does not exist for Burundi: the violence mainly occurred in 1972 and 1988, within the same time series. For symmetry's sake, I have included the former data series.

The Rwandan figures give no indication of the death and fleeing of hundreds of thousands of people (around 10% of the country's then 2.6–2.8-million population) at the beginning of the 1960s (Fig. 1, top). With annual population increments at the time in the range of 60,000 people, incidents such as the killing of up to 10,000 in 1963 and the fleeing of approximately 100,000 in 1960–61, should be clearly visible. Instead, both datasets indicate that population increase continued exactly as before. In the case of Burundi, the data do indicate a dip in annual population increases at the beginning of the 1970s. Yet the data show this dip to be a small,

slow, decade-long, incremental evolution. In reality, hundreds of thousands of people (up to 10% of the country's overall population) either died or fled Burundi in May and June 1972 — none before, few after. These people did not flee or die over a decade. The other outbreaks of violence (1965, 1988, 1991) are undetectable in the population data.

Thus, the UN population data of both Burundi and Rwanda fail to reveal the deaths and displacements. In the case of Rwanda, the data completely hide any increased mortality or sudden massive emigration; in the case of Burundi, they present a low, spread-out impression of the decline in population, instead of the sudden spasm of extreme violence that occurred. How has this come about?

The United Nations adjusts population data that are provided to it by governments. As the 1992 *FAO Production Yearbook* explains it:

The United Nations *Demographic Yearbook* gives time series of estimates on total population. These data are generally provided by the countries, but in some instances the U.N. Statistical Office adjusts the available estimates or prepares new ones. However, for many developing countries, further adjustment of available estimates is needed in order to maintain a reasonable degree of consistency. . . . Therefore, the U.N. Population Divi-

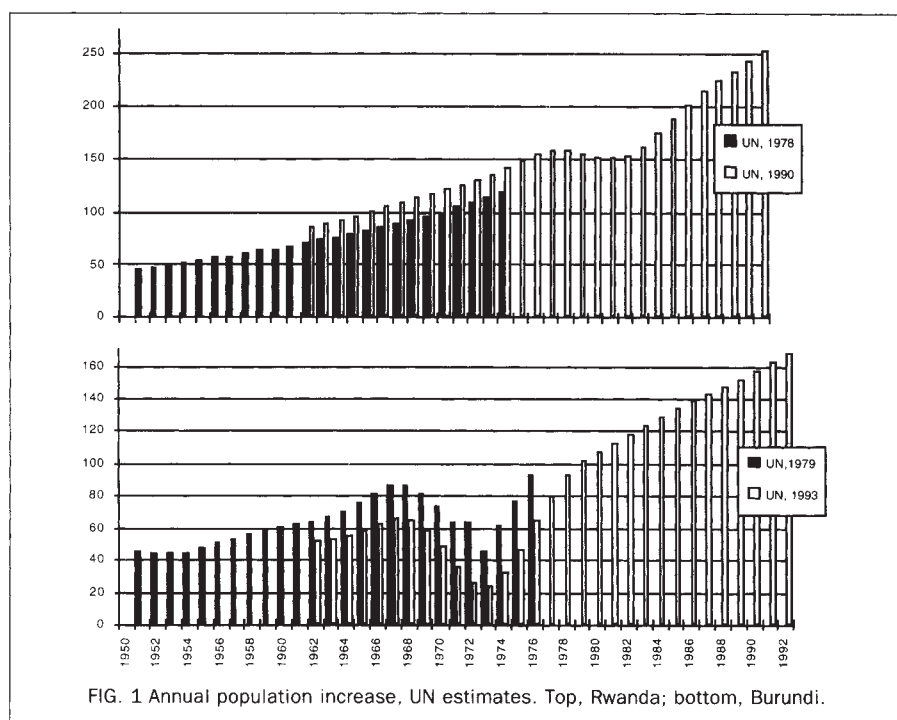


FIG. 1 Annual population increase, UN estimates. Top, Rwanda; bottom, Burundi.

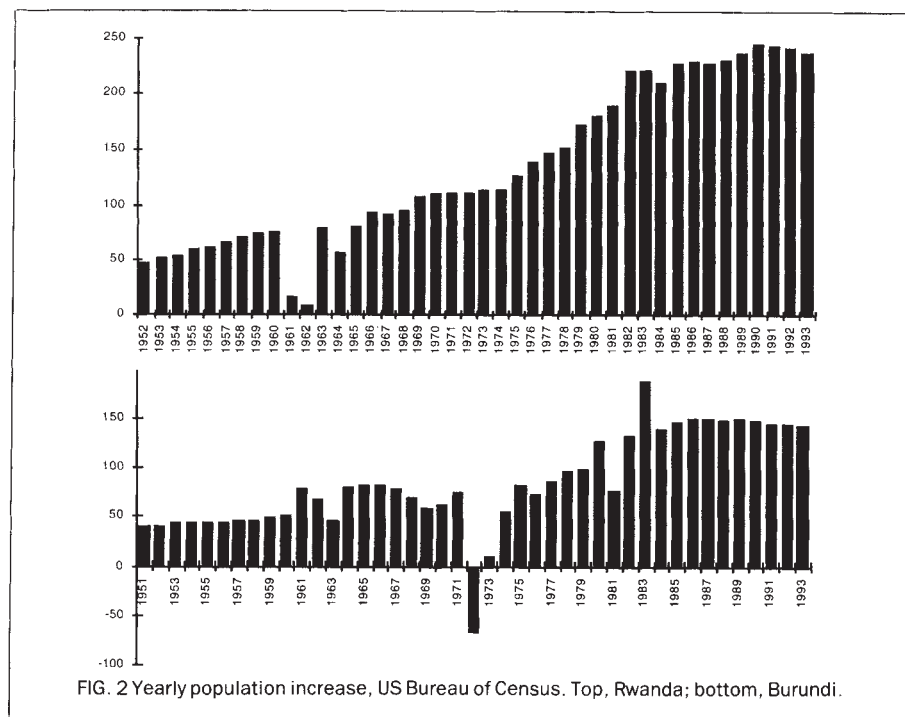


FIG. 2 Yearly population increase, US Bureau of Census. Top, Rwanda; bottom, Burundi.

sion prepares a complete series of estimates for each country covering a fairly long period. The data presented in this table are derived from these series. . . . The total population series are revised by the United Nations periodically and the series presented in this table are based on the latest assessment⁷.

(The table referred to above is Fig. 1 here.) Hence, there are two active agents in the creation of population data: the governments of the countries concerned, and the UN Statistical Office.

The governments of Rwanda and Burundi have always refused to acknowledge the massive killings within their boundaries, executed largely by their military apparatus. Hence, it comes as no surprise that they would also seek to cover up these events in their population data. That is all the easier for them to do as there are no past hard, 'incontestable' data: censuses are far between, very partial, and made by government officials. Rwanda, for example, had some very partial censuses in 1952 and 1970. It then published no new data, forcing the United Nations either to make its own estimates or to publish no data. Burundi had a small, very partial census in 1970–71. That gave it the opportunity to start a new series in 1972 (rendering the new data incomparable with the earlier data), which it continued until 1977. For both countries, the first reasonable census was not conducted until 1978–79. Until then, the United Nations temporarily presented its own estimates, indicating that they were unreliable or non-comparable⁸.

The UN Statistical Office has the mandate and the competence to adjust or revise the data so as to correct gaps and errors. Somehow, however, this seems

not to have happened on these occasions, for Fig. 1 clearly does not reflect reality and hides the genocides that occurred. How has this come about?

A combination of two factors is at work. First, UN officials, like all demographers, start from the last, more-or-less reliable, available census, then work backwards to the previous census (or forwards to the next one), using models that include variables for fertility, mortality and emigration. The 1978–79 full censuses in both Burundi and Rwanda were considered more reliable than the partial censuses of the past, and so all population data were recalculated. Thus, all presently available data have been constructed after 1979, 10 to 20 years after some of the main occurrences of violence. To the relatively junior UN official who would have calculated the new data series in the beginning of the 1980s, there may have seemed no urgent reason to bother to account for past instances of violence. Second, the United Nations produces its population estimates only on a 5-year basis; the years in between are simply interpolations of these benchmarks. This means that, as far as aggregate population data are concerned, one cannot observe the impact of occurrences of violence in the year it occurred, but only at the next 5-year cutoff point. For Rwanda, there is still no indication of the 1959–63 events in the 1965 data (nor in 1970), nor is there of the 1988 or 1989 events in Burundi. The 1975 Burundi cut-off point, however, is lower than what would have prevailed in the absence of the 1972 violence. The United Nations did, therefore, account for part of the 1972 Burundi killings, but in the unrealistic manner presented in Fig. 1.

It could be argued that everybody knows that population data for all African countries, especially unstable ones, are unreliable. The logic thus follows that Burundi and Rwanda are two typical examples of this, and so the UN data are simply the best that can be expected. But although it is true that African governments lack the logistical, financial and administrative capacity to produce high-quality censuses, it is false to argue that the UN population data are just examples of this general fact of life. For one reason, the data presented above are not slightly off the mark: they bear little resemblance to the real world and hide important events. Second, at least one other institution produces population data — the US Bureau of Census — that presents a very different picture, clearly reflecting occurrences of violence (Fig. 2). These data are not necessarily more accurate: indeed, they suffer from the same lack of reliability as all other estimates. Yet they show that genocide can be accounted for.

In conclusion, it is clear that African population data — as well as all other macro-economic and social data — are at best informed estimates. Yet even so, it is surprising that the UN data, which are authoritative and widely used, are so unrealistic as to hide some of the worst genocide the world has witnessed since the Second World War. As we have seen, there are understandable technical reasons for this state of affairs. This argument is not only a purely 'moral' or political one. Indeed, most of the important indicators for development are of a 'per capita' nature: GNP, industrial production, exports, or debt per capita to calculate economic growth, food production or calories per capita to monitor hunger, and so on. As scientists, we like to compare trends in these data with each other, or with other factors, such as introduction of new technologies or adoption of new policies. Clearly, the relevance of these exercises is seriously compromised by the inaccurate UN data. □

Peter Uvin is at the Alan Shaw Feinstein World Hunger Program, Box 1831, Brown University, Providence, Rhode Island 02912, USA.

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ACKNOWLEDGEMENTS. I thank W. Bender, K. Benefo, A. Demmke, T. Fowler, E. Messer, M. Morris and S. Cu-Uvin for discussions.

Cycles of growth and destruction

Recent advances in understanding of the eukaryotic cell cycle and its disruption in cancer have been dramatic. Participants at *Nature's* conference "Cycling out of control" (Paris, 1-2 December) found that they continue.

READERS of *Nature* will have noted several papers about the ecology of Scandinavian voles over the past few years. These rodents undergo marked swings in population, reproducing at very high rates in good years, only to suffer catastrophic losses as their food supply runs out and their predators multiply. While the details of this process have puzzled theoretical ecologists for some time, it is clear that in an ecosystem, at least, unfettered reproduction has its limits.

Within a multicellular organism, things are not so simple. In this case, a cell whose replication proceeds unchecked will shortly kill the organism from which it sprang, along with itself. The very behaviour characteristic of all organisms from bacteria to blue whales, to multiply as fast as one's surroundings will permit, is here disastrous. So the cells of metazoan animals have grafted a stringent set of checkpoints onto the cycle that directs eukaryotic cell division.

Of course, that cycle is itself carefully controlled, so that a cell that has replicated its DNA cannot do so again before mitosis, and a cell that has not cannot undergo mitosis. In fission yeast, the latter block is imposed by *cdc18*, a protein made late in G_1 which is essential for DNA replication and prevents mitosis until it is complete (Paul Nurse, ICRF, London, whose epic performance when faced with a slide projector which obstinately refused to recognize his slides will not soon be forgotten by anyone present). But what prevents mitosis before then? The answer appears to be a protein made early in G_1 called *rum1*, which is a potent inhibitor of mitotic cyclins. These cyclins themselves have the opposite role: they are made only in G_2 , and prevent replication unless preceded by mitosis.

More details of the process have emerged from studies of budding yeast, where the activity of mitotic-type cyclins called *C1b5* and *6* (which are, confusingly, necessary for DNA replication) appears to be held in check by a protein called *p40^{Sic1}* (Kim Nasmyth, Institute of Molecular Pathology, Vienna). The latter is earmarked for destruction at the appropriate moment by a ubiquitin-conjugating enzyme encoded by the *Cdc24* gene, but only after *Cdc6* (the budding yeast equivalent of *cdc18*) has triggered the assembly of replication complexes. In *Drosophila* and *Xenopus* embryos, these controls appear to be added progressively during development, as the initial rapid cycles are slowed by the introduction of refinements such as G_1 and G_2 (Pat O'Farrell, University of California, San Francisco, and

Tim Hunt, ICRF, South Mimms, England).

Onto this base, metazoan cells add a programme that directs them to die rather than proliferate when damaged or in response to an inadequate signal. This pathway can be blocked by the protein *bcl-2*. But whereas there is apparently only one such protein in *Caenorhabditis elegans*, it has numerous relatives in mammalian cells, all of which can form heterodimers with at least one other family member, and some of which actually promote apoptosis (Stan Korsmeyer, Washington University, St Louis). Some viral proteins have similar properties. The 19kD E1B protein of adenovirus may even improve upon *bcl-2* by protecting the lamins of the nuclear envelope, which are broken down in apoptosis (Eileen White, Rutgers University).

But perhaps the most important protein inactivated in cancer is *p53*, which directs cell-cycle arrest or apoptosis in response to DNA damage. The E6 gene of oncogenic human papilloma viruses, for instance, inactivates *p53*, increasing the frequency of DNA rearrangements by up to five orders of magnitude, without changing cellular morphology or doubling time (Thea Tlsty, University of North Carolina).

Exactly how DNA damage activates *p53* is still unclear. In budding yeast, damage is apparently detected by DNA polymerase ϵ , which signals through the kinases *Sad1* and *Dun1* to initiate the repair process (Steve Elledge, Baylor College of Medicine, Houston). But however the process works in metazoans, *p53* is the commonest gene lost in frank neoplasia, and its absence markedly accelerates gross chromosomal rearrangement (Andrew Wyllie, University of Edinburgh). Cells in this condition are also very resistant to ionizing radiation and cytotoxic drugs, and the discovery that *taxol*^{*}, at least, can induce apoptosis by a route independent of *p53* is therefore particularly welcome (Scott Lowe, MIT).

Other systems may also be inactivated in the majority of cancers. The retinoblastoma protein, *Prb*, sequesters the transcription factor *E2F* (needed for progress through S phase) until it is inactivated by a complex of cyclin D and *cdk4* or *6*. This in turn can be inactivated by the small inhibitors *p15* or *p16*, effectively blocking the cell cycle. Many oncogenic viruses encode *pRb* inhibitors, and up to 40 per cent of tumours have no functional copy of the gene. But it is now

clear that in other malignancies, there are defects elsewhere in the pathway, including (in at least one case of familial melanoma) the gene for *p16* (Ed Harlow, Massachusetts General Hospital, Boston).

The importance to an emergent cancer of preventing apoptosis is underlined by the phenotype of mice lacking the *ras* GTPase-activating protein *GAP*. So far from promoting cancer by activating *ras*, the knock-out is in fact lethal *in utero*, causing massive apoptosis throughout the embryo (Tony Pawson, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto). Other *ras* targets may control cellular behaviour, influencing membrane ruffling and the activity of the related GTPase *ral* (John Cooper, Fred Hutchinson Cancer Research Center, Seattle).

The extracellular signals transmitted by *ras* eventually trigger transcription, but the pattern can be unexpectedly complex. Expression of the proto-oncogene *fos*, for instance, appears to depend on the concerted action of four transcription factors, all of which are necessary *in vivo* (Tom Curran, Roche Institute of Molecular Biology, Nutley, New Jersey). Even the response to a simple rise in cAMP levels depends on a balance between stimulatory and inhibitory forms of the transcription factor *CREM*, which in hamsters directs rhythmic behaviour in addition to its role in the cell cycle (Paolo Sassone-Corsi, CNRS, Strasbourg).

Only seldom is the link between an extracellular signal and the cell cycle obvious. *TGF β* is an honourable exception, as it appears to block cycling by inducing transcription of the *cdk* inhibitor *p15* (David Beach, Cold Spring Harbor Laboratory, New York). But Beach also presented experiments which indicate that the kinase *raf*, once activated by *ras*, can activate some forms of *cdc25*, itself a major activator of cyclin/*cdk* complexes. In some cases, therefore, the route from signal to cycle may be more direct than expected.

Such a remarkable result immediately suggests a new set of genes to screen for oncogenic alterations in cancer biopsies. Even before this is done, however, it is clear that recent advances in our understanding of the cell cycle have revolutionized our view of cancer. Loss of proper checkpoints, allowing resistance to apoptotic signals and an increased rate of mutation, can be seen as a hallmark of the condition. It will be exciting to watch as this and future advances are converted into improvements in patient care.

Nicholas Short

^{*}*Nature* has been informed by Bristol-Myers Squibb that "taxol" has been a trade name registered in that company's name since 1992.

Death of a synapse

Charles Jennings

CURRENT wisdom has it that the developing nervous system emerges by a process of selection, in which initially random synaptic connections are preserved or eliminated according to their patterns of activity. The best-studied example of this phenomenon is the neuromuscular junction, and on page 519 of this issue¹, Balice-Gordon and Lichtman describe how synaptic transmission determines the fate of neuromuscular synapses in the mouse. Their paper not only represents a major technical achievement, it also provides the most detailed account to date of the mechanism by which synaptic connections are eliminated.

In a newborn mouse, each muscle fibre is innervated by several motor neurons. Over the next few weeks, however, most of these connections are withdrawn, until each fibre is innervated by only one motor neuron. Synapse elimination seems to be a competitive process, which in some way involves synaptic activity. But the system has been difficult to study, mainly because there has been no way of following the fate of a single synapse during the elimination period. To circumvent this problem, several years ago Lichtman and colleagues developed methods for staining and repeated examination of individual synapses *in vivo*². Using this technique, they were able to observe a highly specific pattern of elimination, in which all the terminals from one axon were eliminated while adjacent terminals from other axons remained unaffected³.

Prediction

Suspecting that this specificity might be related to patterns of synaptic activity, Balice-Gordon and Lichtman predicted that if synaptic transmission could be altered, the maintenance and elimination of synapses would also be disrupted. The latest paper confirms that prediction.

The authors blocked synaptic transmission using α -bungarotoxin, which binds almost irreversibly to the acetylcholine receptors (AChRs) on the postsynaptic membrane. By applying the toxin locally, they could block transmission at some terminals while leaving others unblocked. They found that local blocking of AChRs causes both the blocked receptors themselves and the overlying nerve terminals to disappear, over a period of several weeks. Remarkably, only those terminals that lie directly over the blocked receptors are eliminated; others, only a few tens of micrometres away, which originate from the same axon, remain unaffected.

What happens if AChRs are blocked throughout the entire junction? In this case — in contrast to a local block — none

of the terminals is eliminated. Elimination of terminals cannot, therefore, be due either to nonspecific effects of the treatment or to a loss of activity *per se*; rather, it requires inactive regions on the postsynaptic membrane to be juxtaposed with other regions that remain active.

The conclusion to be drawn from these results is that synaptic transmission by AChRs produces a signal that tends to eliminate synaptic terminals. Terminals are protected from this signal, however, if they can activate their own underlying AChRs. An active synapse therefore will not eliminate itself, but will eliminate any inactive synapses nearby (see figure).

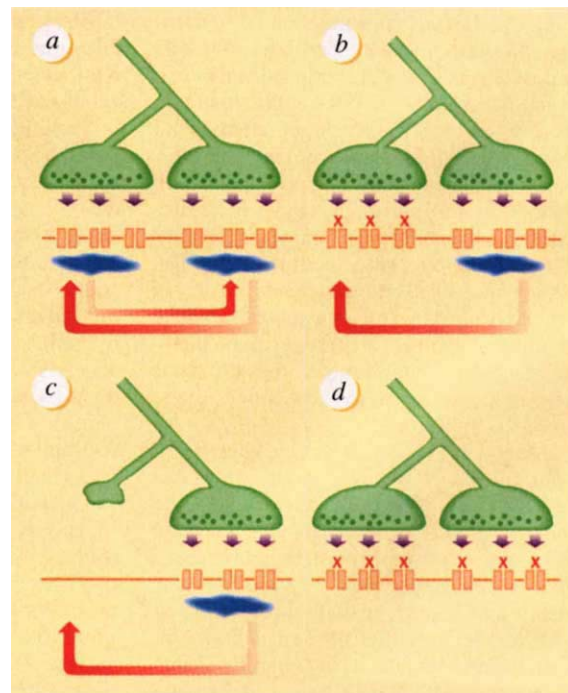
The complete suppression of synaptic transmission by α -bungarotoxin is, of course, an artificial situation. Could the same mechanism explain synapse elimination during normal development, when terminals are unlikely to be completely inactive? Balice-Gordon and Lichtman propose that it could; if two neurons fire asynchronously, and if the protective effect of activating their own AChRs is short-lived, then each neuron will tend to eliminate the other and competition will ensue. Because the first detectable sign of terminal withdrawal is the disappearance of underlying AChRs, it is tempting to speculate that, as the terminals from one neuron start to lose their AChRs, they become both less able to suppress other terminals and less able to protect themselves. They would thus be caught in a vicious circle that would lead to their eventual demise.

This model corresponds to Hebb's rule, which proposes that, when pre- and postsynaptic cells fire in synchrony, their connections are selectively strengthened. The model has considerable explanatory power, but must nonetheless be treated with caution. The new data indicate that a terminal must activate AChRs in order to protect itself, but they do not show that its activity must be synchronized with that of its competitors. This is a central assumption of the Hebbian model, and it will be important, but probably tricky, to find out whether it holds *in vivo*.

The model does however

receive indirect support from the work of Poo and colleagues^{4,5}, who have described a Hebbian mechanism at frog neuromuscular synapses in culture. When a muscle cell is innervated by two motor neurons, postsynaptic activity evoked by one neuron causes rapid suppression of synaptic transmission from the other unless it also fires in synchrony. To escape suppression, pre- and postsynaptic activity must coincide to within a few tens of milliseconds. Interestingly, the suppression appears to be due to a presynaptic decrease in ACh release, meaning that a retrograde messenger molecule may be involved. It remains to be seen whether these rapid effects on synaptic transmission (which are somewhat reminiscent of long-term potentiation in the hippocampus) have any relationship to the much slower morphological changes observed by Balice-Gordon and Lichtman.

It is clear from the new findings that AChR activation causes both elimination and protection of synapses. But what might be the basis for these distinct effects? The elimination signal seems to travel at least 50 μ m (because it eliminates adjacent terminals; see figure), whereas the protective signal must be more locally confined (only the immediately overlying terminal is lost when AChRs are locally blocked). The AChR is an ion channel that allows influx of both sodium and



a, In a normal adult neuromuscular junction, each terminal activates its underlying acetylcholine receptors (AChRs), thereby causing an elimination signal (red arrows) and a local protective signal (blue). b, When AChRs under one terminal are blocked with toxin (red crosses), they are no longer protected from elimination, and are unable to eliminate other terminals. c, The unprotected AChRs and their overlying terminal are eventually lost. d, If all AChRs are blocked, no elimination signal is produced, so no loss occurs.

calcium, so one appealing (but speculative) model would be that the suppressive signal is mediated by membrane depolarization, while the protective signal is mediated by calcium influx.

Question

Whatever they are, these activity-dependent signals must originate postsynaptically. The question then is how they can cause the loss, not only of postsynaptic AChRs, but also of presynaptic terminals. A likely target may be the synaptic basal lamina, the thin layer of extracellular matrix that lies between the muscle fibre and the nerve terminal. This structure plays an important role in regulating both pre- and postsynaptic differentiation, and contains several known or putative signalling molecules, including agrin⁶, S-laminin⁷ and ARIA/neuregulin (ref. 8; S. Burden, personal communication); any of these could be a target for activity-dependent regulation. In addition, several intracellular proteins are concentrated under the postsynaptic membrane, where they are thought to help anchor AChRs at the synapse, and these too may be involved⁹. Because Balice-Gordon and Lichtman have made it possible to produce neuromuscular synapse elimination at will, the way is now open to analyse in detail the molecular events that accompany, and perhaps control, this process.

Finally, what of the brain? A large body of evidence indicates that activity can produce changes in synaptic connections throughout the nervous system, and at least some of these changes may have a structural basis (see, for example, ref. 10). It will be technically very demanding to perform on central synapses the type of experiments that Balice-Gordon and Lichtman have carried out on the neuromuscular junction, not least because of the difficulty of relocating a single synapse among the 10¹³ or so within the rodent brain. Nonetheless, although it will be years before we fully understand how the brain develops and maintains its connections, the present study has provided the clearest view yet of how the answers may turn out. □

Charles Jennings is an assistant editor at Nature.

Platinum apples of the asteroids

John S. Lewis

It is always entertaining when a new insight turns our notions of good news and bad news on their head. Such is the case with Jeffrey S. Kargel's analysis, in the *Journal of Geophysical Research*¹, of the economic potential of asteroids in Earth-crossing orbits.

With the exception of a few unrepentantly pure uniformitarian holdouts who maintain that impacts are unlikely, geologists and space scientists now widely recognize that Earth-crossing asteroids and comets threaten the Earth with a 'stick' of monumental proportions. Astronomical images of the recent impact of fragments of the comet Shoemaker-Levy 9 on Jupiter, showing explosions equivalent to tens of millions of megatons of TNT, with dense dust palls covering areas larger than the entire surface area of the Earth, have greatly broadened the acceptance of impacts as a real and important natural force. Indeed, as geological, physical and chemical research on large impacts progresses, it takes less and less imagination to picture an Earth devastated by a comet or asteroid.

Of course, many space scientists were aware of the impact threat long before Shoemaker-Levy 9. The so-called iridium layer left at the time of the extinction at the end of the Cretaceous period has been known for nearly 15 years, since the pioneering work of Luis and Walter Alvarez and their co-workers. Most large asteroid impacts leave a distinctive signature of metallic elements that are normally very rare in the Earth's crust because they were very efficiently extracted into the metallic core at the time of first melting and differentiation of the planet. These elements include not only iridium, but also the entire family of platinum-group elements. Thus the presence of high concentrations of these elements in sediment layers with the same proportions as in meteorites reveals contamination of the Earth's crust by non-terrestrial meteoritic materials. It has long been known that the average near-Earth asteroid (NEA) contains abundant iron, nickel and cobalt, carrying in solid solution much higher concentrations of the platinum metals than the richest known ore body on Earth^{2,3}. Schemes for the extraction and separation of these ferrous and platinum-group metals have been suggested for some years⁴⁻⁶.

In response to our growing awareness of the impact hazard, a number of schemes for protecting the Earth from impactors have been proposed (see especially ref. 7). These proposals range from the simplistic idea of blowing up a threatening asteroid (a frightening and almost certainly fool-

hardy endeavour) to diverting its path by rocket propulsion, nuclear explosions, solar sails, or other means. As the time interval from the discovery of a threatening body to impact is typically 10 million years, and almost always at least a few centuries, there is ample time to carry out the small deflections needed to assure that the body should pass the Earth.

Kargel, of the United States Geological Survey in Flagstaff, Arizona, emphasizes that the large majority of all asteroids, especially those that contain abundant metals, are veritable treasure troves of platinum-group and precious metals. His analysis shows that kilometre-sized metal-bearing NEAs contain up to 400,000 tonnes of these metals, worth about \$5 trillion (5×10^{12} US dollars) at present market prices. Kargel points out, though, that that is not the relevant answer: by means of an economic analysis of supply and demand for rare strategic materials, he estimates that flooding the market with ten times the present global production rate would depress the market value to \$320 billion. Would the enterprise still be profitable?

This production rate is sufficient to 'use up' a threatening kilometre-sized NEA and process it into innocuous (indeed, highly desirable) products in about 20 years. The iron, nickel and cobalt by-products of this extraction operation would themselves be of enormous value for use as construction materials in space, for example, for use in building constellations of Solar Power satellites in high orbits about the Earth that are much more accessible from NEAs than from the Earth's surface⁸. Meanwhile, the threatening asteroid is broken up and chemically digested before it can do harm.

Recent studies of the value of non-terrestrial materials have generally emphasized the use of water, oxygen, hydrocarbons and other materials synthesized on other Solar System bodies as a means of lowering the cost and increasing

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the capabilities of space missions⁹. The propellants and life-support materials made in such a programme are used in space, not returned to Earth. Thus the most avidly desired products have been low-tech materials with high demand in space; the cost savings, of course, result from dramatic decreases in the mass that must be lifted (at a cost of about \$20,000 per kilogram) out of the Earth's gravity well. An excellent example of this approach is the synthesis of liquid oxygen and liquid carbon monoxide out of martian atmospheric carbon dioxide to serve as the propellant for an unmanned sample return mission (or, for that matter, a manned expedition)¹⁰.

But Kargel also turns this approach on

its head: he proposes seeking a product with so high a value per kilogram that it would be profitable to return it to Earth. The NEAs are ideal targets for such exploitation, as they have both very high concentrations of valuable materials and very low energy requirements for return to Earth, much lower than for return from the Moon. Thus the 'stick' that threatens Earth is also a 'carrot' of monumental proportions. As so often happens, the line between a disaster and an opportunity is a fine one indeed. □

John S. Lewis is in the Space Engineering Research Center for Utilization of Local Planetary Resources, University of Arizona, Tucson, Arizona 85721, USA.

SIGNAL TRANSDUCTION

Dorsal developments

Philip Ingham

OF the many similarities and parallels between vertebrates and *Drosophila* revealed in recent years, the relationship between T-cell activation and dorsoventral patterning of the fly embryo must count as one of the more curious examples. Establishment of dorsoventral polarity is a highly complex process, beginning long before fertilization and culminating in the generation of a graded distribution of Spätzle activity, a signalling molecule found in the perivitelline fluid that surrounds the developing embryo^{1,2}. The Spätzle signal is then transduced across the egg cell membrane, creating a gradient of activity of the transcription factor, Dorsal, different levels of which activate or repress various patterning genes along the dorsoventral axis³.

Although the proteolytic cascade responsible for generating the Spätzle gradient is itself a fascinating process, it is the transduction of the Spätzle signal that has excited most interest outside the *Drosophila* community. The reason is that Toll, the putative Spätzle receptor, has significant sequence similarity in its intracellular domain to the vertebrate interleukin-1 receptor⁴; and Dorsal is a homologue of the vertebrate transcription factor NF- κ B⁵, activity of which is regulated by, among others, interleukin-1. Of particular interest are the downstream components of the Toll signalling pathway — for it is hoped that analysis of these components will help unravel some of the mysteries surrounding cytokine signalling and NF- κ B regulation. Two such components are the products of the *pelle* and *tube* genes, and on page 563 of this issue⁶ Großhans *et al.* describe a series of experiments that tell us something of how they regulate Dorsal.

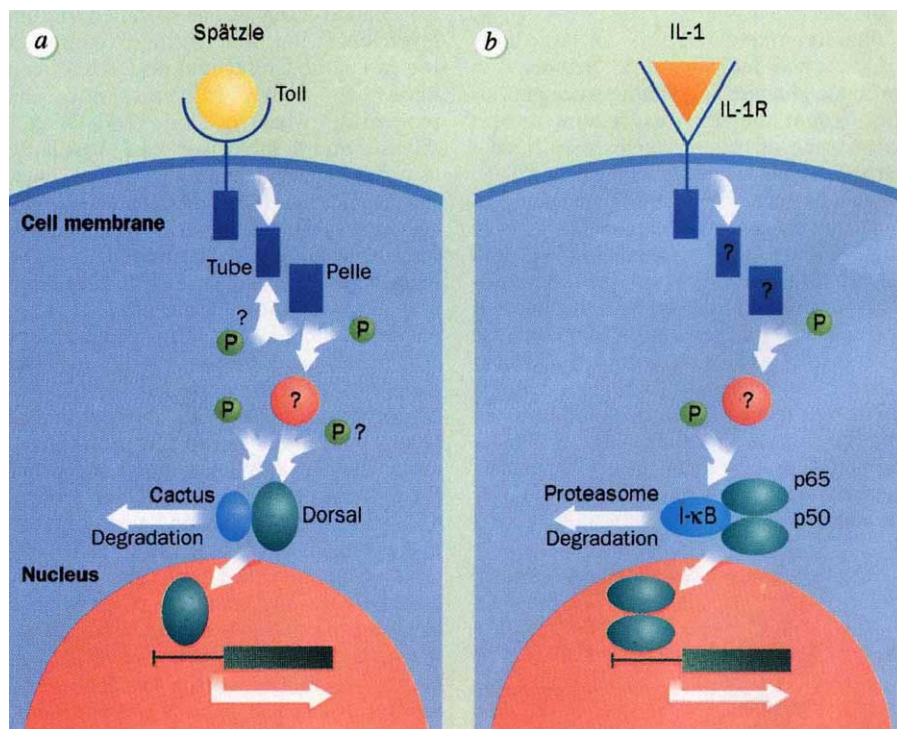
In vertebrates, a major control point for

NF- κ B activity is its association with the protein I- κ B, which acts to mask the nuclear localization signals in the NF- κ B protein, inhibiting its entry into the nucleus⁷. Numerous protein kinases have been implicated in the activation of NF- κ B *in vitro*, phosphorylation of I- κ B lead-

ing to dissociation of the NF- κ B-I- κ B complex and suggesting a mechanism by which NF- κ B could be released for nuclear uptake in intact cells⁷.

Studies *in vivo*^{8,9} have however cast doubt on the significance of this mechanism; when cells are treated with specific proteasome inhibitors, NF- κ B is not activated upon stimulation, but remains associated with I- κ B which is nevertheless phosphorylated. These findings suggest that phosphorylation results not in the dissociation of the inactive NF- κ B-I- κ B complex, but rather in the targeting of I- κ B for degradation; it is the degradation of I- κ B which releases NF- κ B, leaving it free to enter the nucleus and activate transcription. This mechanism clearly differs from that by which various other kinases activate NF- κ B *in vitro*, so the identity of the kinases that act *in vivo* remains an open question.

In *Drosophila*, the homologue of the I- κ B protein is encoded by *cactus*^{10,11}, a genetically identified antagonist of Dorsal activity. Double-mutant analysis shows that mutations in the genes *pelle* and *tube* suppress the effects of an activated Toll receptor on Dorsal nuclear uptake; thus both genes seem to act downstream of Toll to regulate the Cactus-Dorsal interaction. Whereas the product of *tube* is a protein of unknown biochemical function¹², *pelle*



a, The Pelle serine/threonine kinase is activated by Spätzle activity, probably by direct interaction with the Tube protein which forms a link between Pelle and the activated Toll receptor protein. Activation of both Pelle and Tube may involve membrane association or dimerization (or both). Pelle then acts either directly, or through an intermediary, to promote the nuclear uptake of Dorsal via the phosphorylation of Dorsal and/or Cactus. b, An attractive hypothesis suggested by studies in mammalian systems is that phosphorylation of Cactus targets it for proteasome-dependent degradation. Degradation of the Cactus homologue I- κ B has been shown to release NF- κ B p65 and p50 for nuclear uptake. Whether homologues of Pelle and Tube are responsible for regulating this process in vertebrate cells remains to be seen.

encodes a serine/threonine kinase¹³; but although the implication is seductive, whether or not Pelle could act directly to phosphorylate Cactus protein has remained obscure because the sequence in which the two genes act has until now been unknown.

Großhans *et al.*⁶ now describe how they have generated dominant activated forms of both proteins by fusing their coding regions with the extracellular and transmembrane regions of the Torso receptor kinase. As predicted, either of these activated forms is sufficient to activate Dorsal in the absence of activated Spätzle; but whereas Tube function depends upon Pelle activity, the reverse does not apply. This places Pelle downstream of Tube in the Toll–Dorsal pathway, the appropriate position if Pelle is the kinase responsible for phosphorylating Cactus.

Life and nature are seldom that simple, however, and *in vitro* assays show that Cactus is only feebly phosphorylated by Pelle. Dorsal protein is phosphorylated to a similar degree while — paradoxically — Tube proves to be a major substrate for Pelle activity: and, in line with this, direct association between the Pelle and Tube but not the Dorsal or Cactus proteins is demonstrated by the yeast two-hybrid assay, as well as by immunoprecipitation. The functional significance of this activity remains to be established *in vivo*, but one possibility suggested by the authors is a feedback step that regulates the signalling pathway.

These studies make a strong case that Tube acts as a direct regulator of Pelle. But, as the authors concede, the role of Pelle itself remains enigmatic — certainly, their data do not rule out the possibility that it phosphorylates Cactus — and its potential to phosphorylate Dorsal is consistent with the results of genetic analyses. Cloning the vertebrate homologues of the *tube* and *pelle* genes should help in understanding cytokine signalling pathways, but it seems certain that many other components remain to be discovered in both systems. □

Philip Ingham is at the Imperial Cancer Research Fund, PO Box 123, Lincoln's Inn Fields, London WC2A 3PX, UK.

SQUIDS cross a watershed

John Clarke

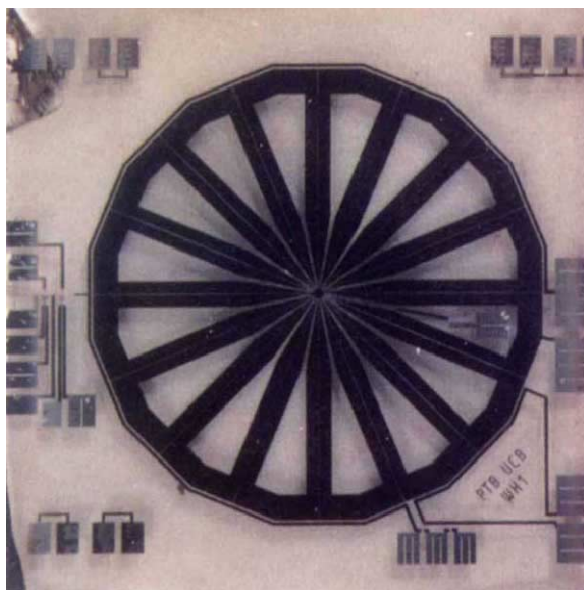
GEOPHYSICS, magnetocardiology and magnetoencephalography — all demand sensitive measurements of magnetic field. Currently, the best bet is to use a low-transition-temperature (low- T_c) superconducting quantum interference device, or SQUID, operating in liquid helium. But such remarkable progress has been made in the technology for SQUIDS based on high- T_c superconductors that, somewhat to the amazement of those in the field, we now find ourselves with devices that are good enough for magnetocardiology and on the verge of what is needed for application to magnetoencephalography. The drive has been to produce ever quieter, more reliable SQUIDS that could be operated in liquid nitrogen; and at a meeting in Boston*, a surprisingly large number of participants reported such SQUID-based magnetometers with noise levels low enough to supplant their liquid-helium-cooled counterparts in many applications. High- T_c SQUIDS are, it seems, ready for the real world.

SQUIDS, which have been the most widely used low- T_c superconducting devices for almost 30 years, come in two varieties. The first and more common is the d.c. SQUID, which consists of two Josephson junctions connected in parallel on a superconducting loop and operates with a static bias current. When the magnetic flux threading the loop changes, the voltage across the device oscillates with a period of one flux quantum (Φ_0). Suitable electronics convert a small voltage change into a current which feeds back into a coil coupled to the SQUID to nullify the applied flux. In low- T_c devices, typical noise levels are $10^{-6}\Phi_0 \text{ Hz}^{-1/2}$ ($10^{-6}\Phi_0$ in a 1-Hz bandwidth).

The second type, the radiofrequency SQUID, involves only one Josephson junction and operates with a flux bias ranging from 20 MHz to 10 GHz. Although SQUIDS are exquisitely sensitive to magnetic flux, their small area implies that they are not particularly sensitive to magnetic field (that is, flux per unit area). Generally, the effective area and hence the sensitivity are enhanced with a superconducting flux transformer, to achieve noise levels as low as a few

femtotesla per square-root hertz.

To make high- T_c devices, films of the superconductor $\text{YBa}_2\text{Cu}_3\text{O}_{7-x}$ (YBCO) are grown in the presence of O_2 by an *in situ* process — usually excimer laser deposition or sputtering — so that the films grow stoichiometrically and epitaxially with their *c*-axis perpendicular to the



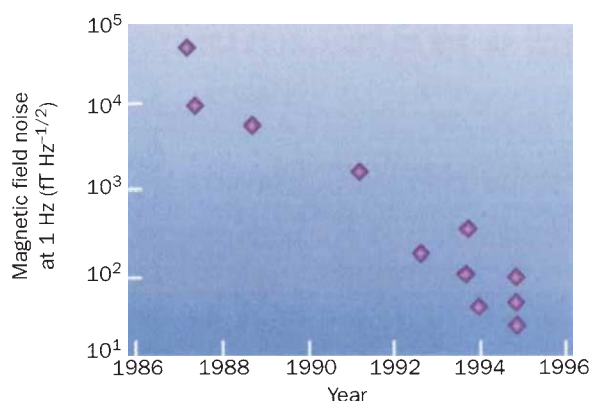
A 16-turn, multilayer magnetometer. Device is 7 mm across. (Berkeley, PTB Berlin, Univ. Birmingham.)

substrate, which is heated to about 800 °C. In Boston, most authors described magnetometers with grain-boundary Josephson junctions, depositing the film across either the grain boundary of a SrTiO_3 bicrystal or a step edge that had been ion-milled on a single crystal of SrTiO_3 , LaAlO_3 , MgO or yttria-stabilized zirconia.

The magnetometers fall into two classes: those requiring a single layer of YBCO and those involving multilayers of YBCO and insulating films. Several groups reported 'directly coupled magnetometers' in which a YBCO film is patterned by photolithography to form a d.c. SQUID coupled to a large pickup loop. A magnetic field applied to the pickup loop induces a supercurrent which is directly injected into the SQUID. For instance, one such device on a $20 \times 20 \text{ mm}^2$ bicrystal achieved $26 \text{ fT Hz}^{-1/2}$ at 1 Hz (R. Cantor, Conductus Inc., Sunnyvale). Taking a different tack, Y. Zhang (Forschungszentrum Jülich) described a radiofrequency SQUID with a large-area washer that is 'flipped' over onto a separate chip bearing a single-layer YBCO flux transformer, so that the two devices are inductively coupled. With a $40 \times 40 \text{ mm}^2$ pickup loop, the noise of this 'flip-

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*1994 Applied Superconductivity Conference, Boston, 16–21 October 1994.



How the magnetic field noise at 1 Hz (fT Hz^{-1/2}) has improved with time for SQUID magnetometers operating in liquid nitrogen at 77 K.

chip' magnetometer is 24 fT Hz^{-1/2} at frequencies down to 0.5 Hz.

Multilayer devices are more difficult to make but offer the prospect of higher sensitivity for a given area. One kind of magnetometer involves a flux transformer with a large-area pickup loop connected to a smaller multilayer coil inductively coupled to the SQUID (again, in a 'flip-chip' arrangement). The best of these devices achieved noise levels of about 75 fT Hz^{-1/2} at 1 Hz (F. Ludwig, Univ. California, Berkeley, and Lawrence Berkeley Lab., with a 100 mm² loop, and M. N. Keene, DRA Malvern, with a 430 mm² loop). Fully integrated devices in which the coil is deposited directly on the SQUID were also popular. Yet another integrated multilayer device is the 'fractional turn magnetometer' in which the SQUID consists of a number of loops (usually 8 or 16) connected in parallel; the best performance was 37 fT Hz^{-1/2} at 1 Hz, with a device only 7 mm in diameter (Ludwig and colleagues at Univ. California, Berkeley, Physikalische-Technische Bundesanstalt Berlin, and Univ. Birmingham). Finally, several planar gradiometers — measuring an off-diagonal derivative of the magnetic field — were reported, including one with a resolution of about 100 pT m⁻¹ Hz^{-1/2} at 1 Hz (Keene).

Because many applications require low noise at frequencies around 1 Hz, it was particularly noteworthy that the level of the infamous 1/f noise ('flicker noise') at low frequencies f continues to drop dramatically (see figure). The cause is a steady improvement in the crystalline quality of the films and the adoption of an electronic noise cancellation technique.

This progress bodes well for applications in the near future. Already, in Boston, we saw remarkable magnetic images obtained by scanning a sample next to a high- T_c SQUID (R. C. Black, Univ. Maryland; O. V. Snigirev, Moscow State Univ.). Such systems have great possibilities for nondestructive evaluation (A. Cochran, Univ. Strathclyde; Black).

M. Klinger (Forschungsgesellschaft für Informationstechnik, Bad Salzdetfurth) reported a high- T_c SQUID 'spinner magnetometer', in which one rotates a geological sample to determine its remanent magnetization. A. H. Miklich (Univ. California, Berkeley) described a picovoltmeter, and a Berkeley-Conductus collaboration a three-axis magnetometer for geophysical application. Impressive magnetocardiograms were obtained with a 16-channel magnetometer (H. Toyoda, Superconducting Sensor Laboratory,

Sumitomo Electric and Electrotechnical Laboratory).

Now that high- T_c SQUIDS have come of age, the simple fact that liquid nitrogen boils away much more slowly than liquid helium will make them accessible to a much broader range of users. Of course, work still needs to be done. Particularly for that holy grail of biomagnetism —

imaging the heart and brain — still more sensitivity may be needed.

At the same time, for clinical applications it will be essential to develop systems that operate without an expensive magnetically shielded room to attenuate ambient magnetic field fluctuations. One approach is the magnetic gradiometer, which discriminates against distant noise sources with relatively small magnetic field gradients in favour of nearby signal sources with higher gradients. Electronic cancellation among sensors (for example, A. A. Fife, CTF Systems Inc., Port Coquitlam, British Columbia; R. H. Koch, IBM, Yorktown Heights) further reduces the ambient noise. Despite these challenges, particularly now that a user-friendly high- T_c SQUID magnetometer with a performance competitive with the best research devices is available off-the-shelf (Conductus's iMAG), one need not be too cautious in predicting a rapid growth of applications by the next conference in 1996. □

John Clarke is in the Department of Physics, University of California, Berkeley, California 94720, USA.

SUPERCONDUCTIVITY

New warmth at 1 K

Zachary Fisk

A ROBUST undercurrent of the strong and continuing effort over the past 8 years in high-critical-temperature (high- T_c) superconductivity has been the discovery of a surprisingly rich variety of new and unusual superconducting materials with low to modest T_c . This includes the fullerenes, (Ba,K)BiO₃, boro-carbides and heavy fermions. Now, elsewhere in this issue, we have a report of the first non-cuprate layered perovskite — Sr₂RuO₄ — that is superconducting near 1 K (Y. Maeno *et al.* *Nature* 372, 532–534; 1994). This T_c will not excite those driven solely by the technological applications of superconductivity, but it is another story with those still trying to understand the cuprate materials at the fundamental level.

Cuprate superconductivity remains arguably the problem in condensed matter physics. All the big questions about these materials which were raised by the original discovery by Bednorz and Müller remain. What is the superconducting pairing mechanism? How are these superconductors different from conventional ones? What is special about the CuO₂ planes present in all cuprate superconductors? And is the quasi-two-dimensional character of the cuprate materials fundamental to the physics of this superconductivity?

This is not to say that we have not learned an enormous amount both about

cuprate superconductivity and the cuprate materials more generally, both from experimental and theoretical studies. But the phenomena we study do not arrive unaccompanied: to isolate what is crucial to the physics from its irrelevant clothing is the central task. With materials as unusual as the layered perovskite cuprates this is strange clothing, and this is why the discovery of a copper-free superconductor with the same structure holds high promise for new and real progress in high- T_c research.

Extensive research has gone into looking for non-cuprate analogues to the high- T_c materials, with no success until now. In fact Sr₂RuO₄ was rather heavily worked over, motivated by reports (from work pre-dating the discovery of high- T_c compounds) that it had metallic as well as antiferromagnetic properties. However, Maeno's group finds that properly prepared single crystals are not antiferromagnetic; rather, they show superconductivity below 1 K, and show resistance anisotropy above this T_c very similar to the high- T_c cuprates. Instead of CuO₂ planes, we have here RuO₂ planes.

It is a part of the established lore of superconductivity that crystal structure is idiosyncratically relevant to superconductivity: certain structures are favourable for superconductivity, yet nobody has any

real idea why. Quasi-two-dimensional as well as oxide-based superconductors have a much longer history than the cuprates, but with this new layered oxide compound we now have the first directly relevant superconductor for comparison with the high- T_c cuprates. This, surely, should give us some chance to factor out the structural peculiarities of the layered perovskites.

Where do we stand now in understanding high- T_c superconductivity? Not at a consensus by any means. Although there is a majority that favours an electronic rather than a phonon mechanism for the superconducting pairing of electrons, the latter still has its adherents. We now have detailed theoretical predictions based on particular models, for example on magnetically driven pairing. There are also interesting and provocative arguments that question whether the concepts developed over the past 70 years for the description of metals are adequate for understanding even the normal-state properties of the cuprates.

On the experimental side, there is growing but not yet definitive evidence for a non-trivial superconducting order parameter. These same experiments can be done on the new ruthenate material, and there is even some advantage that the T_c is so low: 1 K is a much quieter environment physically for exploring details of a phase transition than 100 K. These experiments should tell us a lot. There is no *a priori* reason to expect the superconductivity to be qualitatively the same in the ruthenate and isostructural cuprate; in fact it is reasonable to guess that the superconductivity of the ruthenate is 'conventional'. But we need experiment to tell us what the signature of 'conventional' is in such materials, and the detailed comparison of physical properties between ruthenate and cuprate provides a handle on the problem which has been lacking until now. It is for experiment to show theory the ground it must walk on.

Thanks to the intractable character of the high- T_c problem, experimentalists are beautifully tooled up, as never before, with probes that can be used to advantage on low- T_c non-cuprate materials. There is an enormous amount to learn: our understanding of real materials is little more than phyllostatic and being seriously outstripped by the discovery of ever more exotic ones. We can surely expect other ruthenates analogous to the zoo of cuprate compounds. The kind of clean comparison that appears possible with this discovery fuels new enthusiasm in (to cite Hermann Weyl) our "fight to wring significant facts from an inflexible Nature, who says so distinctly 'No' and so indistinctly 'Yes' to our theories". □

Zachary Fisk is in the Department of Physics, Florida State University, Tallahassee, Florida 32306, USA.

Killing the sex ratio

Olivia P. Judson

INFANTICIDE has probably been practised by many different cultures throughout human history¹. Where it has occurred, it is likely to have been biased against females. In recent years, sex preselection, prenatal sex determination and sex-selective abortion have facilitated the process of delivering offspring of the sex of choice, and in many countries the availability of such techniques has led to increased abortion rates of female fetuses². The social costs of sex selection — including abductions, forced polyandry and child marriages — are likely to be great; further, no one knows the genetic or demographic consequences of a consistent cultural bias towards one sex.

In a paper in the latest issue of *Theoretical Population Biology*³, Kumm and colleagues tackle this second question. Using mathematical models to explore the interaction between cultural bias and the genetics of human sex ratios, they conclude that a strong cultural bias may have profound consequences for the sex ratio.

In general, in human societies today, slightly more boys are born than girls. But — in the absence of cultural bias — slightly more boys die at all ages^{4,5}. The usual explanation follows that of Fisher, who predicted that the sex ratio would be evolutionarily stable at 1:1. This is easy to see. If a population had many more males than females, any individual that produced only daughters would, on average, have more grandchildren than individuals that produced sons or sons and daughters. The tendency to produce daughters would spread until the sex ratio returned to 1:1, at which point there would no longer be an advantage in producing daughters.

Fisher went on to argue that the ratio of male to female offspring at the end of a period of parental investment should reflect the differences in cost of rearing males and females. If one sex is more costly to produce than the other, then either the sex ratio at conception (known as the primary sex ratio), or the sex ratio at the end of parental care, or both, may differ from 1:1. According to Fisher, in humans, boys are 'cheaper' to conceive, but more 'expensive' to rear; he wrote that "the actual sex-ratio in man seems to fulfil these conditions somewhat closely . . . since this adjustment is brought about by a somewhat large inequality in the sex ratio at conception, for which no *a priori* reason

can be given, it is difficult to avoid the conclusion that the sex-ratio has really been adjusted by these means"⁴.

As modern human populations age, the adult sex ratio generally becomes skewed towards females. But in societies where infanticide, neglect or abandonment of daughters is common, the adult sex ratio may be heavily skewed the other way^{2,5}.

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Single aim: a poster pushes China's one-child policy.

For example, in some areas of China, India and Pakistan, the skew has been reported to be as high as 5:1 towards males⁶. Kumm *et al.* show that the effect that this may have on the primary sex ratio depends on whether there is a fitness cost to infanticide — that is, it depends on whether parents who practise infanticide have, on average, fewer children than those who do not.

If infanticide does carry a fitness cost, then any infanticidal parent whose genes favour the desired sex will have a greater fitness than an infanticidal parent whose genes do not. In this case, the primary sex ratio will evolve to become biased towards the preferred sex; if the cost of infanticide is sufficiently high, the primary sex ratio may become distorted rapidly. On the other hand, if infanticide does not carry a fitness cost, then the primary sex ratio should become biased against the desired sex or, as Fisher predicted, the sex ratio should shift towards the scarcer sex.

Kumm and colleagues use gene-culture coevolution models, a class of mathematical models that has been developed within the past 20 years, as a tool to try to understand the interaction between genetics and culture^{7,8}. Many models of this type stem from population genetics and have been expanded to treat a cultural tendency as a trait that may be passed from parent to offspring. Such models will often be built from simple assumptions, but their behaviours may be complex.

One of the crucial assumptions underlying models of infanticide is that there is genetic variation in the sex ratio. This notion has been disputed, with some claiming that a sex ratio of 1:1 is merely the consequence of constraints exerted by the sex chromosome systems⁹. Indeed, evidence for genetic variation in mammalian sex ratios is limited, and attempts to manipulate the sex ratios of agriculturally important animals have been largely unsuccessful.

Nonetheless, different human populations do seem to show primary sex ratios that are biased to different extents. Most human populations have a primary sex ratio that is biased towards males, but in China and India the primary sex ratios are significantly more male-biased than in other societies^{3,10}. Male-biased sex ratios could arise if for some reason — for example, grown-up sons contribute more than grown-up daughters to their parents — sons are a less costly investment than daughters¹¹. But both China and India¹² have a history of preferring male children; the primary sex ratio may reflect a recent bias towards sons that carried a large fitness cost. In China today, the one-child policy means that parents who favour one sex are no longer likely to suffer a fitness cost. If the current bias against daughters continues, the primary sex ratio in China should start to become more and more

female biased; as this happens, the discrimination and sex selection required to maintain the desired sex ratio will increase.

Thus, Kumm and colleagues draw two sobering conclusions. First, the nearly universal bias of the primary sex ratio towards males may reflect generations of past infanticide against females. Second, current practices of sex selection may interact with genetics to produce increasingly pronounced distortions in the primary sex ratio. □

Olivia P. Judson is in the Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK.

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NITRIC OXIDE

More jobs for that molecule

Solomon H. Snyder

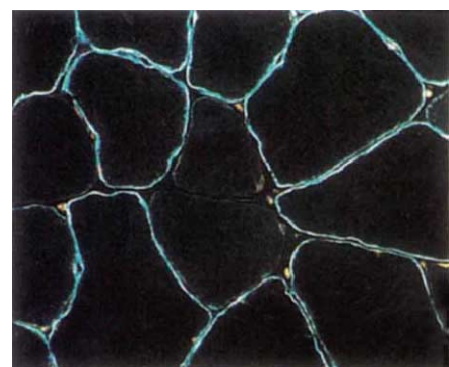
It is not so long ago that biologists became interested in nitric oxide (NO), but in that short period it has been found to carry out more important functions than virtually any other known messenger molecule^{1,2}. As they report on page 546 of this issue³, Kobzik *et al.* have found yet further tasks for this versatile marriage of nitrogen and oxygen. Their paper contains a sizeable body of data detailing the effects of NO in rat skeletal muscle.

Biologically, NO was first characterized as endothelial-derived relaxing factor, being formed by endothelial cells in blood vessels and diffusing to the adjacent smooth muscle to cause vasodilatation. Endogenous formation of NO is now regarded as the main determinant of blood pressure, and the principal drugs used in treating angina act by being converted to NO. About the same time macrophages — white blood cells that engulf and kill tumour cells and bacteria — were shown to act by forming large amounts of NO when stimulated by endotoxin.

Subsequently, the molecule has been identified as a neurotransmitter in the

brain and peripheral autonomic nervous system, where it mediates a large number of biological functions ranging from learning and memory to peristalsis and penile erection. These three principal sites of NO formation are paralleled by the existence of three distinct forms of NO synthase (NOS) derived from distinct genes.

Neuronal NOS, the first to be molecularly cloned, possesses numerous regulatory sites, including a critical binding site for calmodulin which enables NOS production to be induced by stimulation of glutamate *N*-methyl-D-aspartate (NMDA) receptors that open calcium ion channels. Excess release of NO may be responsible for severe neurological damage in vascular stroke, as NOS inhibitors block NMDA neurotoxicity as well as stroke damage². Moreover, mice in which the gene for neuronal NOS has been deleted manifest much less brain damage following vascular stroke than wild-type mice⁴. Endothelial and neuronal NOS enzymes are generally regarded as constitutive, with increases in enzyme activity being triggered by stimuli that increase calcium influx. By contrast, the mac-



Digitized photomicrograph showing muscle fibres stained for nitric oxide synthase. Type I fibres are stained; type II are not.

rophage enzyme is inducible⁵ — in their resting state macrophages have no detectable NOS, but stimulation by endotoxin triggers a massive increase in synthesis of the protein.

But these are stereotypes. Following the initial characterization of the three main subtypes of NOS and NO functions, a second wave of NO research is rapidly overturning them. For instance, it has turned out that the 'non-inducible' neuronal NOS is in fact inducible. Following damage to peripheral or central neurons, high concentrations of neuronal NOS are detected in neurons that normally lack the enzyme^{6,7}. During embryonic development, major transient expression of neuronal NOS occurs in a neuronal pathway proceeding from the cerebral cortex to the thalamus, in olfactory neurons and in sensory ganglia⁸.

Macrophage NOS is not expressed only in macrophages, but in many other tissues as well; indeed, it seems possible that an inducible macrophage-like NOS occurs in virtually all tissues as a primitive sort of immune system¹. Endothelial NOS does not occur only in blood vessels. The enzyme is also concentrated in a variety of neuronal structures in the brain, especially the pyramidal cells of the hippocampus, where it may provide the NO postulated to serve as a retrograde messenger in long-term potentiation because these cells lack classical neuronal NOS⁹. Finally, neuronal NOS is not confined to neurons, in that it has been detected in the epithelium of the respiratory tract¹⁰.

Kobzik and associates³ now add to the list. They describe the existence of neuronal NOS in skeletal muscle, where it is concentrated in fast fibres and seems to have a physiological role in relaxing muscle. Last year, neuronal NOS messenger RNA was identified in samples of human skeletal muscle by Nakane *et al.*¹¹, and Kobzik *et al.* have followed up on that result by employing immunohistochemical techniques to localize the enzyme primarily to type II fast-contracting muscle fibres. Enzyme activity and immunostaining are concentrated in the mem-

CARBON CYCLE

brane fraction of muscle fibres (see figure), challenging another NO catechism that holds that neuronal and macrophage NOS are always cytoplasmic, while only endothelial NOS is associated with cell membranes.

What does skeletal muscle NOS do? Inhibitors of the enzyme increase skeletal muscle contraction, effects that are reversed by NO donors, indicating that NO presumably physiologically relaxes skeletal muscle in much the same way that it relaxes the smooth muscle of blood vessels³. So, how might it bring about this effect? In blood vessels NO binds to haem in the active site of guanylyl cyclase to augment its activity with the newly formed cyclic GMP, eliciting relaxation by some undefined mechanism. Evidence for some involvement of cGMP in NO activity in skeletal muscle comes from observations that 8-bromo cGMP elicits modest relaxation, as does an inhibitor of phosphodiesterase, whereas an inhibitor of guanylyl cyclase causes contraction³.

Kobzik *et al.*³ suggest another intriguing way in which NO might regulate skeletal muscle. They observe release of NO but not of reactive oxygen intermediates in uncontracted skeletal muscle, whereas in contracting muscle they detect high levels of the reactive oxygen intermediates (presumably comprising superoxide and possibly other oxygen free radicals). It is well known that NO binds to superoxide, thus inactivating it. On the other hand, the combination of NO and superoxide can form peroxynitrite which decomposes to hydroxide free radical, one of the most reactive of all free radical species. One can speculate that NO modulates the effects of oxygen free-radical species formed by contracting muscle, though whether NO diminishes or enhances such effects is unclear. Conceivably, during states of great hyperactivity of muscle, hydroxyl free radicals formed from NO and superoxide could lead to muscle damage — in such circumstances, NOS inhibitors might display therapeutic actions. □

Solomon H. Snyder is in the Department of Neuroscience, The Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, Maryland 21205, USA.

Vanishing in Bermuda

J. R. Toggweiler

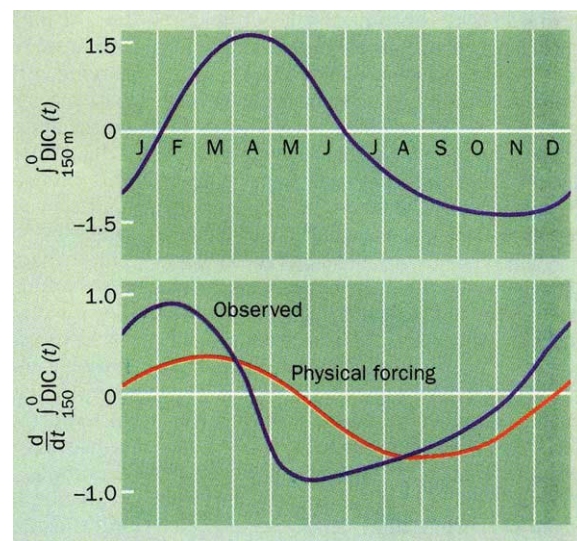
Six years ago the Bermuda Atlantic Time Series station (BATS) was set up to document changes in the carbon, nutrient and productivity cycles at a single point in the open ocean. Measurement capabilities there have increased to the point where a complete suite of carbon system parameters is measured at least once a month. Photosynthetic carbon uptake and the downward flux of biogenic carbon in sinking particles is measured, along with fluxes of carbon dioxide across the air-sea interface. On page 537 of this issue, Michaels *et al.*¹ report on the first two years of carbon system results. They find a repeating seasonal cycle in which dissolved inorganic carbon (DIC) stocks rise during the late autumn and winter and decline during the spring, summer and early autumn.

This was largely expected. What was not expected is that only 20 per cent of the decline during the spring and summer period can be accounted for in the flux of biogenic carbon in sinking particles. The authors rule out most of the obvious unmeasured carbon sinks and conclude that most of the carbon drawn down in the spring and summer has somehow escaped detection.

The upper few tens of metres of the ocean constitute what oceanographers call the 'mixed layer'. It is illuminated by light from the Sun and is stirred by the wind. In a place like Bermuda it is devoid of the nutrients nitrate and phosphate for most of the year. The seasonal cycle helps pump carbon and nutrients through the mixed layer as follows. Winter cooling overcomes the stratification at the base of the mixed layer and allows the mixed layer to deepen (to as much as 200 m at Bermuda). Inorganic nutrients and old respired carbon from subsurface layers become entrained into the mixed layer as it deepens. Although the nutrients are welcome to photosynthetic algae, the deep mixing is not. When mixing extends to 200 m these single-celled organisms do not remain in the illuminated upper layers long enough to maintain high rates of photosynthesis.

Warming of the upper layers during the spring restores a shallow mixed layer. Photosynthetic organisms suddenly find

themselves in a well-lit environment along with nutrients left over from winter mixing. They manufacture new pigment and reproduce rapidly, converting inorganic carbon to organic carbon in the process. Some of the fixed carbon finds its way into particles that are large enough to sink.



Top, sketch of the seasonal anomaly in dissolved inorganic carbon (DIC) in the ocean near Bermuda. Units are moles m^{-2} . The DIC anomaly is integrated over the top 150 m of ocean. Bottom, time derivative of the observed vertically integrated DIC anomaly (purple), plotted together with a sketch of the physical forcing effect on DIC (red). Units are $mol\ m^{-2}\ month^{-1}$. The forcing curve shows mainly the effect of seasonal temperature changes on the solubility of CO_2 ; its mean value is set slightly below the zero line to reflect the fact that the North Atlantic is a net carbon sink because of its thermohaline circulation.

This leads to a downward carbon flux into subsurface layers where the particles decompose. Inorganic CO_2 and nutrients from the decomposition thus become available for the next cycle.

The area around Bermuda was originally thought to be an ideal place to budget the carbon cycle. It comes as quite a shock that the system should be so leaky. Michaels *et al.* suggest two alternatives: either the sediment traps which are designed to measure the sinking flux are simply missing 80 per cent of the flux, or else physical advection is dominating the budget in ways that were not foreseen. The first hypothesis implies that the biological carbon export at Bermuda is fairly substantial: some 30 to 40 per cent of all the carbon fixed by photosynthesis would have to be exported to explain the spring and summer drawdown of dissolved inorganic carbon.

The best evidence that the sediment traps are deficient comes from levels of ^{234}Th in the water column. This isotope is

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a short-lived (24-day half-life) daughter of ^{234}U which has a strong affinity for particles. A level of ^{234}Th activity in the mixed layer that is less than the activity of the parent implies that some of the ^{234}Th has become attached to particles that have sunk out of the mixed layer on a timescale short with respect to its half-life. ^{234}Th provides a fairly direct way of measuring the efficiency with which sediment traps capture the vertical flux.

During the spring of 1993 the amount of ^{234}Th caught in the sediment traps at Bermuda was substantially less than the amount expected given the ^{234}Th deficit observed in the mixed layer. This implies that the sediment traps did indeed miss part of the organic carbon flux. To convert ^{234}Th information quantitatively into a carbon flux, one must know the ratio of organic carbon to ^{234}Th in the sinking particles that were not caught. There are a lot of pitfalls here, but Michaels *et al.* show that the sediment-trap hypothesis closes the carbon budget only if a high ratio of organic carbon to ^{234}Th is used. So this hypothesis probably explains some of the budget problem, but not all.

The second hypothesis, that of advection, has some attractive features. The temperate latitudes of the North Atlantic are well known as an area where the ocean's thermohaline circulation influences the carbon cycle². Warm water advected north from the tropical Atlantic loses a tremendous amount of heat to the atmosphere just north of Bermuda before sinking into the deep sea at even higher latitudes. The cooling process leads to a net uptake of CO_2 by the ocean. This CO_2 uptake could be part of the carbon budget at Bermuda if the process produces a CO_2 deficit at the right time of year.

In the upper panel of the figure, I have sketched out the seasonal cycle of DIC based on the two years of data used by Michaels *et al.* The levels peak between the beginning of March and the beginning of May, and reach a trough in November and December. In the lower panel I have sketched the time derivative of this DIC curve. DIC levels increase most strongly in January and February and decrease most strongly in May and June.

The same panel shows the seasonal cycle of the physical forcing on DIC levels at Bermuda. Part of the physical forcing is due to changes in temperature, and part is due to the large-scale thermohaline circulation of the ocean. When surface temperatures are coldest in late winter, the partial pressure of gaseous CO_2 in the water becomes lower than the partial pressure of CO_2 in the atmosphere. This leads to an increase in ocean DIC through gas exchange. Conversely, when surface temperatures are warmest in late summer, CO_2 is lost to the atmosphere. To reflect the effect of the thermohaline circulation, I have displaced the whole physical forcing

curve down slightly with respect to the zero line. The idea here is that the overturning produces a DIC sink which continuously removes carbon from the atmosphere and upper ocean. Recent modelling work by Follows *et al.*³ shows explicitly how the seasonal subduction of water north and east of Bermuda acts to pump CO_2 downwards in the North Atlantic subtropical gyre.

Juxtaposition of the observed time-derivative curve with the physical forcing curve shows how the advection cycle overlies the biological cycle. During the winter the observed curve is well above the forcing curve because respired DIC from past biological activity is pumped upward by the deepening mixed layer. Much more DIC is injected into the upper 150 m by this mechanism than by gas exchange. During April, May and June, the observed curve is well below the forcing curve, because of intense biological carbon uptake during the spring bloom. During the last half of the year the observed curve largely follows the forcing curve. This is a time when the tendency of the physical forcing is to lower DIC levels in the system. According to Follows and co-workers, this results mainly from the input of low-DIC water from the Gulf Stream. This input occurs all year, but the effect is masked during the winter because the ocean is gaining DIC by gas exchange along the path of the flow. The effect of low-DIC Gulf Stream water should be especially pronounced during the summer and early autumn when temperatures are high and there is an overall tendency for the ocean to lose DIC to the atmosphere.

As Michaels and co-workers point out, the best way to resolve this issue is to investigate systematically the effect of advection on DIC levels around Bermuda. If the explanation proposed by Follows and his collaborators³ is correct, one would expect to see water with a relatively low DIC content advecting southwards through the vicinity of Bermuda during late summer and early autumn; in other words, DIC concentrations should increase toward the south such that the effect of advection is to decrease DIC levels at Bermuda. If, on the other hand, sediment traps are missing 80 per cent of the downwards flux, advection is probably acting to increase DIC at Bermuda at the same time that the downwards particle flux is removing it. □

J. R. Toggweiler is at the Geophysical Fluid Dynamics Laboratory, NOAA, PO Box 308, Princeton, New Jersey 08542, USA.

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Healthily hooked

MEDICAL science is constantly announcing new perils in modern life, and new ways of dodging them. The result is an epidemic of little popular remedies: trace elements, vitamins, plant extracts and so on, all offering salvation from some dire medical fate. In a brilliant marketing ploy, Daedalus aims to corner this whole market. DREADCO pharmacists are devising a single pill to contain everything that the medics currently recommend. As fast as new fads and findings emerge, they will be incorporated into its formulation; as fast as they are discredited, they will be removed again. No longer will the dedicated health freak have to scan the papers endlessly for the latest worries and recommendations. He will simply put in a permanent order for the 'Omnipill' in its successive updates, and leave the whole business to DREADCO.

Sadly, the Omnipill cannot encompass bulky nostrums such as dietary fibre. But the current formulation has all the vitamins, some of them in megadoses; essential elements such as iron, zinc and selenium; catechin and aspirin to minimize the risks of heart attacks; silicates to limit aluminium uptake; chelating agents against heavy metals; and a few other things for good measure. It is aimed at serious health worriers. A wider, less obsessive market will be reached by 'Omnitonic', a more jovial liquid version competing with tonic wines and cordials, and those beers that claim to be good for you.

In a further masterstroke of marketing, the Omnipill will be addictive. Users will stay with it not merely from medical prudence, but from subtly reinforced habit. Even more cunning, the addictive agent will be nicotine. The current anti-smoking crusade, says Daedalus, is leaving millions of ex-smokers with an aching chemical void in their lives. Omnipill will fill it — with the full blessing of medical science. For nicotine itself is harmless in small doses, has a valuable stimulant effect, and aids slimming. There is even a claim that it counters Alzheimer's disease. It is smoke that damages smokers. Nicotine is smoked only because it cannot easily be stored in pill form: it is slowly oxidized by air. Smoking vaporizes it on demand from its stable combination in the tobacco. But the Omnipill contains catechin and vitamins C and E, all powerful antioxidants. They will protect and stabilize its nicotine. Omnipill will be the first stable, health-enforcing addictive agent. The British government in particular, currently trying to nag its citizens into healthy habits, should welcome it with open purse. David Jones

Fullerenes in Allende meteorite

SIR — The detection of fullerenes (C_{60} and C_{70}) in deposits from meteor impact¹⁻³ has led to renewed interest in the possibility that fullerenes are present in meteorites. Although fullerenes have not previously been detected in the Murchison and Allende meteorites⁴, the Allende meteorite is known to contain several well-ordered graphite particles which are remarkably similar in size and appearance to the fullerene-related structures carbon onions and nanotubes⁵. We report here that fullerenes are in fact present in trace amounts in the Allende meteorite.

The fullerenes were detected in

impact deposits¹ and the residues collected from the Long Duration Exposure Facility² formed as a result of impact synthesis, but comparable to the levels reported in sedimentary deposits associated with the Cretaceous/Tertiary impact event³, possibly produced by extensive wild fires. Because there is no evidence of any shock event in the Allende meteorite's history, the fullerenes found in this meteorite were probably not the result of impact synthesis. Failure to detect fullerenes in previous studies of meteorites⁴ could be because of the well-known inhomogeneous distribution

duced under conditions involving many competing processes; the presence of hydrogen need not preclude fullerene production in the interstellar medium.

In some interstellar environments, hydrogen is present at $H/He < 10^{-9}$, levels much lower than the mean cosmic abundance⁹. Fullerenes can form in the outflows from WC stars¹⁰, for example, which consist mainly of He and C and little or no hydrogen. In fact, the presence of hydrogen may lead to the conversion of fullerenes, C_{60} , to fulleranes, $C_{60}H_n$, resulting in a wide range of molecules from fully aromatic to fully aliphatic, which could explain the variability observed in interstellar/stellar spectra.

L. Becker,

J. L. Bada

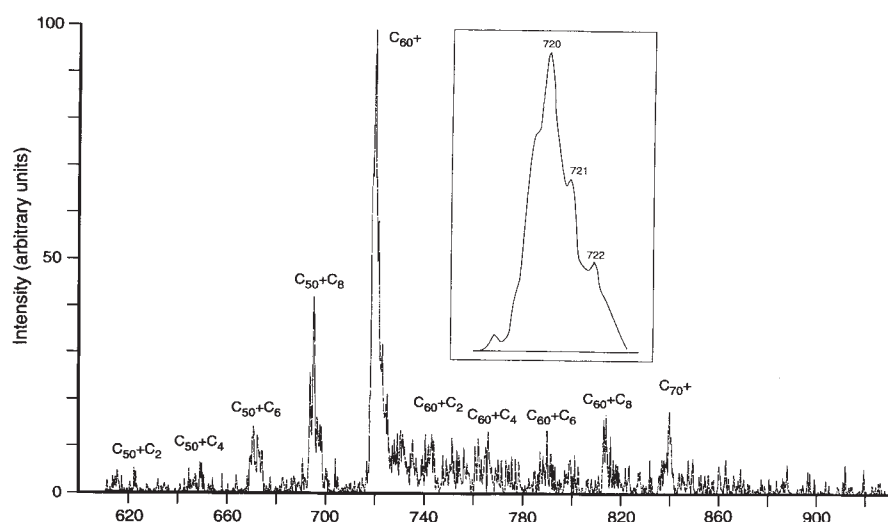
*Scripps Institution of Oceanography,
University of California at San Diego,
La Jolla, California 92093-0212B, USA*

R. E. Winans

*Chemistry Division,
Argonne National Laboratories,
Argonne, Illinois 60439-4831, USA*

T. E. Bunch

*Space Science Division,
NASA Ames Research Center,
Moffett Field, California 94035-1000,
USA*



Laser desorption (reflectron) mass spectrum from sample 15/21. A major peak occurred at $m/z = 720$ (C_{60}^+) and a less prominent peak at $m/z = 840$ (C_{70}^+). Fragmentation peaks from C_{52}^+ to C_{58}^+ and C_{62}^+ to C_{68}^+ were observed at power levels much higher than those originally used to detect C_{60}^+ and C_{70}^+ in the Allende extracts. The insert of sample 15/21 shows C_{60} resolved into 3 peaks at 720, 721 and 722 AMU ($m/\Delta m = 300$), corresponding to $^{12}C_{60}^+$, $^{12}C_{59}^{13}C^+$ and $^{12}C_{58}^{13}C_2^+$, respectively. Sample preparation and analysis of the Allende extracts using a KRATOS (reflectron) time-of-flight instrument is described in detail elsewhere¹. Sample 15/21 was obtained from the Smithsonian Museum (USNM 3529, split 15 position 21).

separate Allende samples by laser desorption (linear and reflectron) time-of-flight mass spectrometry. The observed C_{60}^+/C_{70}^+ peak ratio is 12 (see figure). The C_{60} content for Allende was estimated to be 0.1 p.p.m., considerably less than the fullerene concentrations reported for both the 1.85-billion-year-old Sudbury

of organics within meteorites⁶ or perhaps because fullerenes are present at concentrations too low to be detected previously.

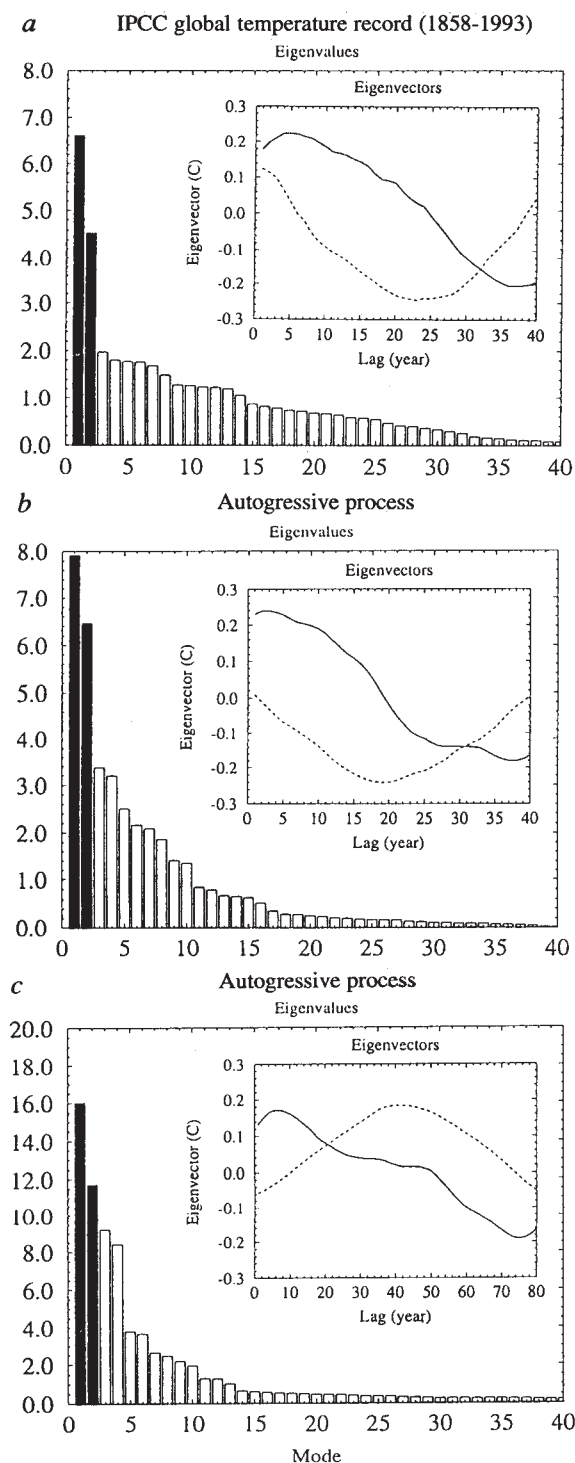
In addition to fullerenes, we detected many polycyclic aromatic hydrocarbons (PAHs) in the Allende meteorite, consistent with previous reports⁶. In particular, we detected benzofluoranthene and corannulene ($C_{20}H_{10}$), five-membered ring structures which have been proposed as precursors to the formation of fullerenes in the gas phase⁷. The observation of fullerenes together with PAHs suggests that PAHs may have been involved in fullerene synthesis, perhaps within circumstellar envelopes or other sites in the interstellar medium. This is somewhat surprising, as such environments generally contain a high abundance of atomic and/or molecular hydrogen⁸. However, the production of fullerenes in sooting flames⁷ implies that fullerenes can be pro-

Low-frequency oscillation

SIR — The singular spectrum approach (SSA) has recently been used to identify a low-frequency oscillation in the global surface air temperature record¹. SSA^{2,3} considers M lagged copies of a series $X(t)$ sampled at equal intervals τ , $X_i = X(t_0 + i\tau)$, $i = 1, N$, and estimates the eigenvalues λ_k and the corresponding eigenvectors ρ_k of their time-lag covariance matrix C , where $1 < k < M$. The eigenvectors are of interest if, among the k eigenvalues, there exist several whose magnitudes are significant. As such, SSA can be used to separate signal from noise.

The figure (a) shows the estimated eigenvalues, λ_k , of the time-lag covariance matrix C (with $M = 40$) for the detrended IPCC global temperature record⁴. Detrending the data is recommended in such cases (when M represents a fairly large fraction of N); otherwise the first eigenvalues representing the trend can mask or alter long-period cycles that might be present. Here we detrend the data throughout by removing the least-squares line. In agreement with the earlier work¹, we observe the first two eigenvalues appreciably higher than the so-called 'noise floor'. These two eigenvalues correspond to eigenvectors (insert in the figure) that exhibit an oscillation of approximately 60–70 years. Eigenvalues far from the 'noise floor' are often

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a, Eigenvalues of the time-lag covariance matrix from the detrended IPCC global temperature anomaly record over the period 1885–1993. The first two eigenvalues extend appreciably above the so-called noise floor and correspond to eigenvectors (insert) exhibiting an oscillation of 60–70 years. b, Eigenvalues of the time-lag covariance matrix from a detrended time series of equal length to the IPCC record and generated from a red-noise stochastic process (see text). As in a, the first two eigenvalues are appreciably above the others and correspond to eigenvectors (insert) exhibiting an oscillation of 60–70 yr. c, Eigenvalues of the time-lag covariance matrix from a detrended red-noise time series of length twice that of the record used in b (272 values). As before, there are two outstanding eigenvalues corresponding to eigenvectors with periodicities of half the record length (insert).

considered to represent deterministic components, whereas all others are assumed to correspond to random processes. As shown below, however, the situation is not always so simple.

The figure (b) shows the eigenvalues and eigenvectors from a detrended time series generated from a stochastic process $X(t+1) = \alpha X(t) + \epsilon(t)$ (first-order linear Markov process) and having the same length of 136 values, where $\epsilon(t)$ is white noise and α the lag-1 autocorrelation of the IPCC temperature record. Signals generated in this manner are simple autocorrelated ($AR(1)$) noise containing no deterministic oscillations.

We observe that b is almost identical to a, indicating that the very low-frequency periodicity of 65–70 years in the IPCC record can arise naturally from the simplest of stochastic processes. The eigenvalues and eigenvector for the same stochastic process but of length 272 (twice the length of the original record) are shown in c. Again we observe two eigenvalues that stand above the rest, and that correspond to eigenvectors with periods commensurate with M . Such spurious periodicities indicate that caution must be used in interpreting eigenvalues associated with low-frequency modes as they may be an artefact of SSA; a proper statistical evaluation must always accompany any evaluation of suspected oscillations in data. Our result suggests that the common practice used in spatial eigenvector analysis of attributing the most significance to the first several eigenvalues is erroneous when applying the SSA.

By decomposing 1,000 detrended $AR(1)$ time series (surrogates) of length 136 and using the same α as the original record, we find that the 99%/1% confidence band overlaps the first two eigenvalues obtained from the data. These results do not change if α is taken from the detrended IPCC record. Although explaining the largest percentage of

variance in the temperature record, these first two eigenmodes (eigenvalue/eigenvector pairs) are clearly indistinguishable from low-frequency eigenmodes of the simplest autocorrelated noise. We note that our confidence limits, obtained by using the eigenvectors of each surrogate series as basis functions (that is, each surrogate has a different set of basis functions), are very similar to the confidence bands obtained by using a fixed, single set of basis functions from the original record⁵. We speculate that the reason for this similarity is that the eigenvectors from the data are not necessarily a better estimate of the population eigenvectors than are the eigenvectors obtained from the surrogates. Indeed, when a bootstrap procedure is applied to the time series residuals (assuming an $AR(1)$ process), we find large error variances for the eigenvector components suggesting that either method of computing confidence limits is appropriate at least for this record.

J. B. Elsner

Department of Meteorology,
Florida State University,
Tallahassee,
Florida 32306-3034,
USA

A. A. Tsonis

Department of Geosciences,
University of Wisconsin,
Milwaukee,
Wisconsin 53201-414,
USA

SCHLESINGER AND RAMANKUTTY REPLY — We have used our physical climate/ocean model and four statistical autoregressive models to simulate the response of the climate system to white-noise forcing, for both the globe and the North Atlantic⁶. Each of the five models is run 1,000 times for the 135 years' duration of the observed global mean temperature record and for the 137 years' duration of the observed North Atlantic temperature record. Singular spectrum analysis (SSA) is performed on the 10,000 simulations after each is linearly detrended.

For the global mean temperature, all the statistical models show statistical significance greater than 89.1%, and the physical model shows a statistical significance greater than 33.5%. For the North Atlantic temperature, all the statistical models show statistical significance greater than 91.3%, and the physical model shows a statistical significance greater than 98.6%. Consequently, the hypothesis that the North Atlantic temperature oscillation is due to climatic noise can be rejected at a very high level of statistical confidence. This hypothesis applied to the oscillation in global mean temperatures can be rejected at a lower level of statistical confidence, this as a consequence of the oscillation not existing everywhere over the Earth¹.

Our results and conclusion differ from those of Elsner and Tsonis because we determine the variance of the white-noise forcing for the physical and statistical models, together with the autoregressive coefficients for the latter, from the observed surface temperature record after removal of the temperature changes contributed by increasing greenhouse gases and anthropogenic sulphate aerosols, that is, after detrending. Elsner and Tsonis determine the white-noise variance and autoregressive coefficient for their statistical model from the nondetrended observed temperature record. Thus, they assume that all temperature changes observed since the middle of the nineteenth century are due to natural variability, whereas we assume that only the temperature changes other than those forced by greenhouse gases and anthropogenic sulphate aerosols are due to natural variability. Their assumption means that, unless the anthropogenic forcing of the climate system has been zero year after year, the sensitivity of the

climate system is zero. Although this would mean that there is no global-warming problem, it is not consistent with the varied palaeoclimatic history of the Earth.

Michael E. Schlesinger

Department of Atmospheric
Sciences,

University of Illinois,
at Urbana-Champaign,
Illinois 61801, USA

Navin Ramankutty

Climate, People and
Environment Program,
Institute for Environmental Studies,
University of Wisconsin-Madison,
Madison 53706, USA

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Glutamatergic signals in *Ephedra*

SIR — Plants frequently synthesize toxic non-protein amino acids that disrupt amino-acid transport, metabolism and protein synthesis in leaf- and seed-eating mammals and insects^{1,2}. *Ephedra foeminea* Forssk. (Ephedraceae, Gnetales), the joint-fir, widely distributed in the eastern Mediterranean and the near East³, contains in its fresh stems substantial amounts of the two *cis*-diastereoisomers of the L-glutamate analogue L-2-carboxycyclopropyl glycine, (2*S*, 3*S*, 4*R*)- and (2*S*, 3*R*, 4*S*)-2-(carboxycyclopropyl) glycine (L-CCGIII and L-CCGIV, respectively)⁴. These two compounds, which constitute about 1% of the stem dry weight in this and a related Mediterranean species, *E. altissima* (see table), are rare. L-CCGIII has been reported⁵ in a few North American species of *Aesculus* (the buckeyes). The presence of L-CCGIV in plants has not been reported before. The stem tissue of *E. foeminea* also contains *cis*-3,4-methano-L-proline, a cyclopropane amino acid previously reported only in *Aesculus parviflora*⁶.

Because L-glutamate is central to

amino-acid metabolism and is a neurotransmitter molecule in vertebrates and arthropods, ingestion of L-2-(carboxycyclopropyl)glycines might alter herbivore behaviour. We tested the membrane activity of three L-CCG isomers on two insect tissues *in vitro*. A Na⁺-dependent glutamate transporter in the beetle epidermis⁷ concentrated both *cis*-isomers, whereas L-CCGIII and L-CCGI (the *trans*-2*S*, 3*S*, 4*S*-isomer found in *Blighia sapida*⁸) caused a cockroach hindgut preparation⁸ to contract. In mammalian neurons, L-CCGIII potentiates responsiveness to L-glutamate⁹ and L-CCGIV activates the N-methyl D-aspartate (NMDA) subtype of the L-glutamate receptor¹⁰.

The stems of many *Ephedra* species also contain substantial amounts of a quinoline, 6-hydroxykynurenic acid (6-HKYNA), known to occur in trace amounts in many plants¹¹. In *Ginkgo biloba*, a primitive tree notably free of insect pests, however, it may constitute 0.24% of the leaf dry weight¹². Similar amounts are seen in *Ephedra* (see table). In the mammalian brain, substituted

kynurenic acids are selective antagonists competing with glycine for binding sites on NMDA and non-NMDA glutamate receptors¹³. 6-HKYNA, however, had no activity on the hindgut, whether applied alone or together with glutamate or quisqualate, suggesting that 6-substituted kynurenic acids are not glutamate receptor antagonists in insect visceral muscle. 7-Methoxykynurenic acid, reported to occur in *E. alata*¹⁴, is not a glutamate receptor antagonist in mammalian neurons¹³.

Most Eurasian species of *Ephedra*, such as *E. distachya* and *E. fragilis*, contain the sympathomimetic drugs ephedrine, pseudoephedrine and pharmacologically related amines¹⁵. Ephedrine in the diet is toxic to the pea weevil². The ephedrine alkaloids and the tannins present in the stems of most Eurasian species are absent from *E. foeminea*, *E. altissima* and *E. foliata* (see table). As methanoproline, too, is restricted to tannin- and ephedrine-free species, we propose that cyclopropyl amino acids act as an alternative form of feeding deterrent. A possible reason why some *Ephedra* species are tannin-free may be that tannins block Na⁺-dependent amino-acid transport, compromising the defensive role of ingested cyclopropyl amino acids. The ephedrine alkaloids found in many *Ephedra* species containing tannins are non-polar and do not need special transporter proteins for their absorption. It is remarkable that, despite the more than 100 papers published over the past 100 years or so on the alkaloid content of *Ephedra*, the presence of cyclopropyl amino acids and 6-hydroxykynurenic acid has previously gone undetected.

Stanley Caveney

Department of Zoology,
University of Western Ontario,
London, Ontario, Canada N6A 5B7

Alvin Starratt

Pest Management Research Centre,
Agriculture and Agri-Food Canada,
London, Ontario, Canada N5V 4T3

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CYCLOPROPYL AMINO ACIDS, 6-HYDROXYKYNURENIC ACID AND EPHEDRINE ALKALOIDS, AND PRESENCE OF TANNINS IN THE FRESH STEMS OF SELECTED *EPHEDRA* SPECIES

Species	L-CCGIII	L-CCGIV	Methano-proline	6-Hydroxy-kynurenic acid	Ephedrine	Pseudo-ephedrine	Tannins
<i>E.foeminea</i>	++++	+++	++	+++	—	—	no
<i>E. altissima</i>	++++	+++	—	++	—	+	no
<i>E. foliata</i>	—	—	++	+++	—	+	no
<i>E. fragilis</i>	++	—	—	++	++++	—	yes
<i>E. distachya</i>	—	—	—	++	—	++++	yes

Amounts of amino acids and alkaloids are expressed as per cent dry tissue weight: (—), not detected; (+), < 0.05 %; (++) , 0.05–0.19%; (+++), 0.2–0.5%; (++++), > 0.5%.

Benefits of food hoarding

SIR — Field experiments of hoarding birds of the genus *Parus* (titmice and chickadees) suggest that stored food is retrieved within a few days after storing^{1–3}. The reason that stored food is retrieved shortly after storing could be to withdraw a temporary food surplus from competitors, or to reduce the time that night energy reserves have to be carried around as body fat.

Another reason for food hoarding could be to increase access to energy during a seasonal decrease in food availability, like in winter. The seasonal pattern in food hoarding among parid birds, with a peak in autumn^{4,5}, is consistent with this. Still, reports that hoarded food makes up part of the winter diet⁴ is no direct evidence for long-term hoarding since it has not been directly observed when that food was actually stored. Here we use a new technique to demonstrate a long-term selfish benefit of hoarding in the willow tit *Parus montanus*. Our results imply that food storing in parids may well have evolved in response to seasonal changes in the food supply.

The risk of pilfering is obvious when foraging areas are shared with conspecifics as in non-kin flocks of many parids⁶. To balance the cost in time and energy spent on hoarding, caches must be retrieved more efficiently than at random search, while the evolutionary stability of such behaviour calls for a recovery advantage for hoarders compared with conspecific scroungers⁷. Demonstration of a high recovery success for hoarders compared

with others simultaneously meets both requirements as conspecifics without prior knowledge of the caching sites should retrieve caches at a random search rate.

We used the radio-ptilochronology technique⁸ to follow the consumption of hoarded food in the field over extended time periods. Bird feathers display growth bars as bands perpendicular to the axis (rachis), with each bar representing one day's growth. Ptilochronology (literally feather time-reading) is the reading of such daily growth bars in tail feathers (rectrices)⁹. Elements ingested while the bird is growing a feather will be incorporated in the current growth bar on the day of consumption. Growth bars containing a radioactive marker can then be identified on an autoradiograph of the feather⁸.

During the winter of 1992/93 and 93/94 we administered food items injected with ³⁵S-containing cysteine to willow tit flocks outside Stockholm, south-central Sweden (59°10'N, 18°20'E) and Arvidsjaur (65°40'N, 19°0'E) in northern Sweden. Earlier all birds had been individually colour-ringed while we removed a rectrix. We provided a particular bird (called the hoarder) with 20 labelled items and the other flock members (called non-hoarders) only with unlabelled items. The provided food items had been labelled with an approximate activity of 5 kBq. The specific activity ranged from 2.9×10^3 to 5.1×10^3 MBq mmol⁻¹.

A minimum of 2 months after provisioning, we recaptured the birds and collected the induced rectrices. With an initial delay of 11 days before the replacement feather protrudes⁸, the induced feathers emerged on average 5.9 days (s.e. = 1.3; $n = 25$) after the labelled food was provided. Subsequent growth then occurs such that growth bars represent an approximate period of 35 days from this on.

The autoradiographs of replacement feathers confirm that hoarders consumed labelled items on 5.1 (s.e. = 0.9; $n = 9$) different occasions per individual as compared with only 1.0 (s.e. = 0.4; $n = 16$) for their conspecific flock mates during the approximately 6 to 40 days after storing when growth bars were laid down, a significant difference ($P = 0.007$, $Z = 2.7$, $n = 9$; Wilcoxon matched pairs). The advantage probably lasted longer as there was no decline in the relative selfish benefit with time (Fig. 2). Also, for one of the hoarders, the regrowth was delayed to approximately 52–86 days after provisioning, during which time this bird retrieved two food items.

Because the bird's memory may not last long enough¹⁰ to account for the selfish benefit in our study, the delay before retrieval raises the question of how caches are retrieved. One possibility is that caches are retrieved over longer time periods by updating the memory through

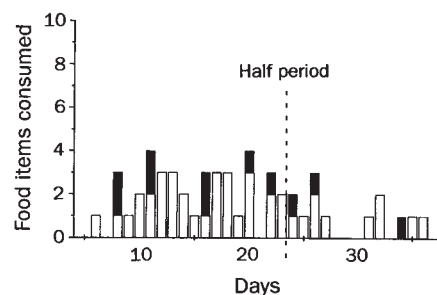


FIG. 2 Ingestion of labelled food during the main period covered by the growing feathers. Replacement feathers emerge after a delay of around 11–14 days⁸, and depending on when we pulled the original feather the induced feathers grew from 6–40 days after storing. Open bars correspond to food consumed by the hoarder, black bars to pilfered items; vertical dashed bar, day 23 (the mid-point of the time window). The recovery advantage for the hoarder compared with pilferers, measured on the total number of ingestions, was very similar before (4.1) and after (4.0) day 23, suggesting that the advantage was not transient. The items retrieved before 6 or after 40 days are not shown.

rehoarding (retrieving and storing in new positions), but the empirical evidence for this is meagre⁵. Individual preferences and exclusive hoarding sites could be other mechanisms securing a recovery advantage. In willow tit flocks there is a spatial separation of feeding and hoarding sites, and this separation is even more pronounced when the flock members choose caching sites¹¹. Hence, with a spatial separation of caching areas the hoarders do not necessarily have to remember the sites to enjoy a recovery advantage.

Anders Brodin

Jan Ekman*

Department of Zoology,
Stockholm University,
S-106 91 Stockholm,
Sweden

* Present address: Division of Population Biology,
Department of Zoology, University of Uppsala, Villavägen 9,
S-752 36 Uppsala, Sweden.

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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. They need not arise out of anything published in *Nature*. In any case, priority will be given to letters of fewer than 500 words and five references.

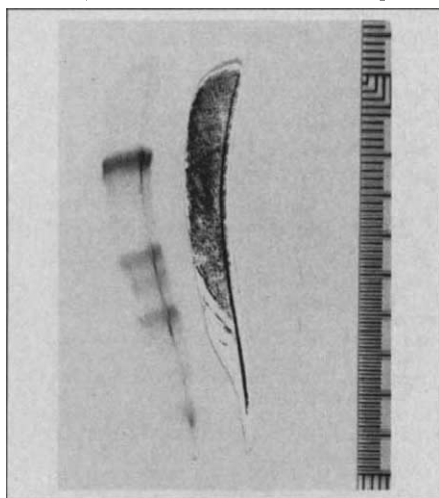


FIG. 1 Autoradiograph (left) and photostat of the outermost left rectrix of an adult male willow tit. The upper limit of the dark band is formed the day the labelled food item is consumed. With a delay of 11 days before the replacement feather protrudes, the actual date of consumption can then be calculated by counting the growth bars (on the photostat) from the tip of the feather.

Of truth and consequences

Tom Gehrels

Wernher von Braun: Crusader for Space. Vol. 1: A Biographical Memoir. Vol. 2: An Illustrated Memoir. By Ernest Stuhlinger and Frederick I. Ordway III. Krieger: 1994. Pp. 375/147 \$42.50/\$29.50.

ON 20 July 1944 a major attempt was made on the life of Adolf Hitler. It failed, but it was clear that high-ranking military officers were involved. The Germans could no longer win the Second World War because of the onslaught by the Soviets from the east and by the Allies from the south and west, as well as the Allies' dominance of the air over the Nazi Reich. Despite all this, hundreds of Germans worked with zest and zeal at Peenemünde, the military rocket-development base on the Baltic coast, and in Dora Mittelbau, the mass-production rocket factory beneath the mountains near Nordhausen, in former East Germany, until nearly the end of the war, in support of Hitler and his *Schutzstaffel* (SS). They did so under the direction of Wernher von Braun, the pioneering rocket engineer. The reasons why they kept working are not explained in Stuhlinger and Ordway's book. Instead, the authors select events to defend and cover up von Braun, continuing to show him at his best until his death in the United States in 1977, when the extent of his wartime involvement began to leak out.

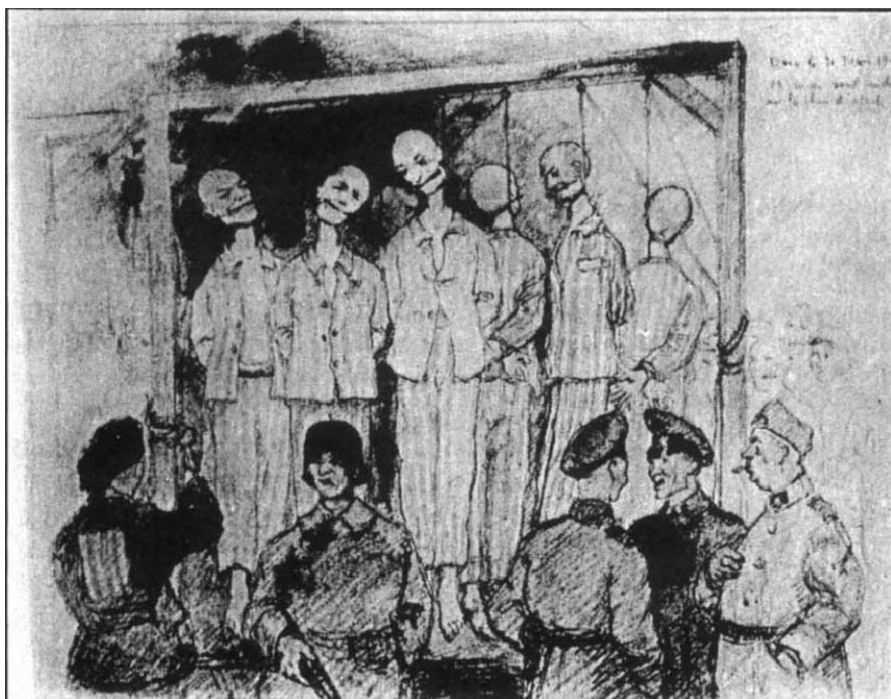
I recommend reading this book as a taste of history. It reminds me of the Nazi propaganda on which the occupied countries were force-fed for years: apparently thorough but selective, appealing but devious — and humourless. There is no new information that could possibly harm von Braun, such as the fact that his brother Magnus was stationed near Nordhausen where the 'Vengeance weapon 2' — the supersonic ballistic V-2 missile used against Britain in 1944 — was being built. Another close associate, Arthur Rudolph, was also at Nordhausen. Troublesome rocket-builders were hanged outside Rudolph's office. The authors disdainfully dismiss them as slave labourers who would have perished in some other concentration camp anyway. Not one photograph of the 60,000 workers appears in the separate volume of illustrations. Nor any of von Braun at Nordhausen, such as those I was shown in 1987 by the director of the museum there (and which, incidentally, are still not on public display).

The authors have missed a great opportunity to describe the drama and ultimate triumph of the human spirit. Among the workers at Nordhausen were some fine human beings, with far more guts and

integrity than von Braun and his cohorts who came from Peenemünde to guide the construction, let alone the supervising henchmen of the SS. That von Braun was a high-ranking SS officer is a remarkable truth that the authors gloss over: they say that his was an honorary position, not mentioning that the SS was a political organization whose members swore a blood oath of allegiance to Hitler and

Nordhausen was Henk van Hoeve, the main supporter of Anne Frank, the Jewish diarist and concentration-camp victim. It was he who supplied Anne and her family with food while they were in hiding in Amsterdam. He was caught by the Nazis soon after they had been betrayed in 1944. Guido Zemsch Schreve was another worker there and is still alive today. Schreve had a daring record of resistance to the Nazi occupation in Western Europe, escaped to England, trained as a secret agent and parachuted back into France. He was also betrayed and ended up at Nordhausen, where he participated in one of the underground resistance groups, whose feats were astounding given the conditions under which they operated.

The challenge for the SS was, of course,



Nazi principles. It seems naive for the authors to emphasize that von Braun was imprisoned by the SS on espionage charges for refusing to cooperate with Himmler over the V-2 project. One almost expects them to have us believe that a magnanimous Hitler father figure released von Braun.

Reading between the lines, one learns that a Faustian pact was made for von Braun's cooperation at Nordhausen. Nobody will be fooled by the excuse that von Braun was working for the "defense of his country". The Germans were the aggressors, seeking to conquer the world for a master race. Von Braun was well aware of this goal: he was raised in an aristocratic family, and his father, who had served as minister of education and agriculture in the Weimar Republic, quit his job when Hitler came to power.

Among the remarkable workers at

how to make such dedicated resistance fighters produce the very best and most intricate war machinery. The security forces achieved this by meting out cruel punishments if they suspected sabotage. Some of the alleged saboteurs were subjects of medical experimentation. Schreve tells how he was bitten on his legs by guard dogs; one leg was treated whereas the condition of the other was left to deteriorate. Men were tortured in a barrack known as the bunker, the prison within the camp where the ever-present *Sicherheitsdienst* (SD or security services) interrogated suspected saboteurs. Their screams were clearly audible from the adjacent parade ground where the barely clad workers stood at roll call for hours on end during that frightful winter. Hangings took place in the early days in the construction tunnel itself and, later, on the parade ground. Dragged to the gallows in

front of their friends, the agonized saboteurs would still manage to find the will to cry out in defiance before they were choked to death. So the Nazis tied little sticks tightly with wire in their open mouths. (These sticks are on display in the museum.) Finally, the bodies were hung above the victims' fellow workers, who, fearlessly and with determination, continued in their sabotage. Of the 5,789 V-2 rockets built, only 2,656 reached their target. Could not — should not — this book have been dedicated to these heroic men, not least to the 20,000 who were murdered?

Von Braun denied any wrongdoing for as long as he could, a front that prevails throughout the book. He is quoted, for instance, as saying that he never saw anyone die at Nordhausen, implying that no one died there. As late as 1975, von Braun and Ordway wrote: "The V-2s took their toll. . . they were responsible for more than 2,500 [Allied] deaths and great property damage". There is no mention of the 20,000 in the Nordhausen area. Stuhlinger and Ordway imply to the gullible reader that von Braun and the other Peenemünde engineers were rarely at Nordhausen. Simple logic, however, shows this to be impossible. The V-2s could not have been built without close supervision by the engineers. And, indeed, later in the book one learns that there were several engineers stationed in the Nordhausen area.

From the US court hearings of Rudolph in 1971 it seems likely that von Braun and his staff had suggested the use of concentration-camp labourers and participated in their management with full knowledge of the grotesquely inhuman conditions and punishments. French workers in the tunnels who are still alive today say that they knew von Braun, Rudolph and other designers by name at the time. Before he died shortly after returning home to Brussels from Nordhausen, Pierre Klein, a tunnel worker, told Schreve how he heard von Braun conclude a detailed inspection with the statement "Daß ist reiner Sabotage [that is clear sabotage]", at which 11 men were hanged.

It took the world a long time to find out what really happened, for who could ever believe such cruelty? Surely any decent human being would have refused to participate in such mass murder, even at the cost of his own life, let alone a scientific achievement.

Von Braun must furthermore share responsibility for prolonging the war. After 20 July 1944, Hitler kept up the Germans' hope and support by promising them a secret weapon. The Allies believed it to be an atomic bomb. Von Braun, however, had learned from Stuhlinger and others that a nuclear bomb programme had been abandoned in 1941. The secret

weapon was his V-2, and he was designing the A9, a longer-range rocket for bombing New York. Von Braun knew that such a weapon could not alter the outcome of the war; it would merely prolong confrontation and human suffering. Here is a war crime even larger than that of the 2,500 killed in the V-2 bombings and the 20,000 murdered at Nordhausen.

So the war ended and the Cold War began. It is all described in the book, including the move of von Braun and his rocket team to Fort Bliss, Texas, to work for the US Army. He was still only 33. In 1950, the team moved to Huntsville, Alabama, where von Braun later became director of the US Army's Ballistic Missile Agency. In 1958 he was chiefly responsible for the launch of the first US Earth satellite and between 1960 and 1970 he was director of the Marshall Space Flight Center, where he developed the Saturn V rocket for the launch of the Apollo craft that landed on the Moon in 1969. Many such facts are presented, but by now the reader wonders how selectively they were chosen and how fairly other pioneers in the space programme are treated. The authors strive hard to show how bright von Braun was and what a nice man he was — a great organizer who was, above all, devoted to his rocketry science. This whitewashing is used even in relating minor instances: it is implied that the astronomer Gerard Kuiper was a supporter of von Braun, whereas Kuiper in fact prevented von Braun from receiving an honorary doctorate, as Stuhlinger knows.

As with all Nazi propaganda, the book is full of clearly erroneous conclusions. The authors have failed to explain their remarkable leader. According to a Nordhausen survivor, it was likely that von Braun's obedience to authority was bred into him by strict schooling. Von Braun was probably too narrowly focused on his rocketry to have seen the Nazi truth and its consequences in the 1930s and to have then left Germany for the United States. Instead, he stuck to his course, and became more and more deeply involved in the military use of his science. The lack of integrity of von Braun and his cohorts is demonstrated by the fact that they never acknowledged what really happened during the war.

The authors' subject matter is an essential part of human history. Time marches on, as do curiosity and science. History should explain our mistakes as well as our accomplishments. Von Braun needs no phony defence, for he was a great man in his own scientific specialization; nor do we need to make hollow excuses for the Americans who indulged him with his rockets and instrumentation. What is needed is a more sophisticated historical perspective, which infuriatingly this befuddling book fails to provide.

Perhaps we can learn from this history. A few nuclear experts today want to use the threat of a collision of a comet or asteroid with Earth to develop new nuclear experiments. US scientists recently visiting a Siberian museum were shown one of the former Soviet Union's nuclear bombs. A question was asked: "Why did you make them?" The answer was, in part: "Because it was such an interesting problem". It is clear from this book that this is what drove von Braun. Should we ask ourselves whether we would have acted any differently from this man had we been born in his place? □

Tom Gehrels is in the Space Science Building, University of Arizona, Tucson, Arizona 85721, USA.

Cassandra versus Pangloss

Stuart Pimm

Scarcity or Abundance? A Debate on the Environment. By Norman Myers and Julian L. Simon. Norton: 1994. Pp. 254. \$21, £16.95.

FOR anyone concerned with the future — ours, our planet's and of science itself — this book will be a compelling read. At its core is a debate held in October 1992 in New York. How panglossian is Simon? "We now have in our hands. . . the technology to feed, clothe and supply energy to an ever growing population for the next 7 billion years". To which Myers replies: "as many as 200,000 generations to come will be impoverished because of what we are doing [now]". From Simon, a professor of business administration at the University of Maryland, and Myers, an Oxford environmentalist, one might not expect compromise. The debate meets this expectation, while yielding fascinating insights into the public's use and perception of scientific knowledge.

This is no ordinary debate, for it cuts to the core of US policy. For Simon, the ultimate resource is people. The more we have, the better off we shall all be, for the more readily we will solve the problems we create. Western-inspired birth-control programmes are inexcusably "racist". Simon delights in getting across this message to the former Reagan administration, which then dramatically cut US aid to developing countries for such programmes. His only fear is the concern that people such as Myers express about the future.

Simon goes first, introducing a long list of examples showing that we've never had it so good and thus the future will be even better. Graphs of life expectancy, infant

mortality, prices of copper and world food production all cheerfully go in the right direction. We are told parenthetically that Africa's food production is lower than it was several years ago, but that "few believe that Africa's suffering has anything to do with a shortage [of resources]". Wars are the cause of suffering but they are not caused by shortages of resources such as oil in the Persian Gulf or land in central Africa. And the environment is becoming cleaner: ambient concentrations of lead, and of DDT (dichlorodiphenyltrichloroethane) and PCBs (polychlorinated biphenyls) in the Great Lakes, are decreasing. Neither Simon nor Myers mentions that these examples are the consequences of bitterly fought campaigns by physicians and environmentalists.

Next, Simon tackles "the statistical flummery about species loss", the "soil erosion scam" and "atmospheric issues". Those who study evolution will find echoes of the attacks on their discipline from religious fundamentalists, in which academic debate about timing and precise mechanisms is interpreted as a large-scale cover-up of evolution's falsity. Similarly, Simon argues that the uncertainties of predicting, say, the degree of global warming is an indication not of the complexity of the problem, but that the problem will simply go away. Certainly, within the careers of key players, concern has gone from global cooling (from increased atmospheric particulates) to global warming, with the eruption of Mount Pinatubo added for confusion. Pleas for future research arise not because the problem is difficult or important but because it generates jobs for scientists, argues Simon. The ozone hole is no problem either — just wear a hat. Indeed, increased ultraviolet radiation may "have beneficial effects" by reducing the incidence of rickets.

Myers's opening statement is devoid of any tables or figures (Simon's contains 24). Simon looks to the past and expects the trends to continue. Myers looks to the best guesses of what the future will bring. Although Simon disputes, distrusts and disparages the claims of scientists, Myers respects them. He starts by quoting the joint statement issued in May 1992 by the US National Academy of Sciences and the Royal Society of London: "If current predictions of population growth prove accurate... the future of our planet is in the balance". Species are becoming extinct in unusual numbers — the experts tell us. The consensus among atmospheric scientists is yes, the climate will warm. Several degrees average difference in annual temperature is not a matter of "spring coming a day or two earlier than usual" but more like the difference between now and when the ice sheet covered half of Britain. As for ozone, Myers might have noticed that

Australian television commercials don't worry about rickets; they urge people to "slip [on a shirt], slap [on a hat] and slop [on the sun cream]".

Questions from the audience follow. Two participants from California and New Jersey feel that life in those places is not incontrovertibly better now than in the past. Los Angeles smog has not obviously decreased. California might be a better place with more people. In the recent election, however, voters in California overwhelmingly supported withdrawing social benefits from those who have crossed the border without visas.

In post-debate statements, Simon criticizes Myers's views on the inevitably irreversible extinction of species. I cannot find anything in Simon's discussion that raises it above incompetence. He argues that if we count perhaps one bird extinction a year then this number is how many species we are losing in total — hardly enough to be of concern. Of course, the world's 10,000 species of birds are less than one in a hundred of the named organisms (and may be as few as one in a thousand of all species named and unnamed). Birds are a typical sample on which to base global extinction rates; one simply multiplies the bird extinctions by the fraction of the planet's species they represent. Even from this sketchy analysis, one quickly gets the extinction esti-

mates of roughly one to several species per day favoured by Myers and his fellow members of the US National Academy (P. Raven, E. O. Wilson, P. Ehrlich, R. May and others). It is hardly difficult to obtain the right order of magnitude, or to see how many orders of magnitude greater it is than extinction rates in the fossil record.

In the post-debate, Myers wins the figures-and-tables battle 14:6. But his best point involves population. Given access to birth control and education, women dramatically reduce the number of children they bear, often to levels of zero growth. Is this a worldwide phenomenon because such education misinforms them?

Two clear questions emerge. If scientists do not find Simon's analyses compelling, nor his dismissal of scientific consensus appealing, then what does this say about their efforts to communicate complex ideas to the public? What do we deduce from the failure of ecology and economics, disciplines with names sharing a common root, to speak mutually comprehensible languages? Science's failure to communicate complex topics is not a new problem, but this book illustrates it as well as any other. □

Stuart Pimm is in the Department of Ecology and Evolutionary Biology, University of Tennessee, Knoxville, Tennessee 37996, USA.

"ALBERT" by Brad Johannsen, a caricature of Albert Einstein lecturing at the Institute for Advanced Study in Princeton, New Jersey. From *Koala: Australia's Ancient Ones* by Ken Phillips, a popular and colourfully illustrated account of this eucalyptus-loving marsupial and its role in mythology. Macmillan Publishing USA, \$27.50.



Carbon capers

Philip Ball

The Most Beautiful Molecule: An Adventure in Chemistry. By Hugh Aldersey-Williams. *Aurum*: 1994. Pp. 340. £18.95.
Perfect Symmetry: The Accidental Discovery of a New Form of Carbon. By Jim Baggott. *Oxford University Press*: 1994. Pp. 320. £18.99, \$29.99.

WHAT should we look for in books about scientific discoveries? James Watson's *The Double Helix* had colourful characters (the author being not the least of them) and the excitement of a race. George Smoot and Keay Davidson's *Wrinkles in Time* had a big question, perhaps the biggest there is. Frank Close's *Too Hot to Handle* had a moral about the scientific enterprise. What is there in an account of the discovery of C_{60} that might illuminate, captivate, entertain?

In detailing the revolution in carbon physics and chemistry that has taken place in the past nine years, both Baggott and Aldersey-Williams hope to capture a certain amount of interest by the sheer beauty of the C_{60} molecule. It is questionable, however, whether this would have had much resonance beyond the chemistry community had the structure not been so easily translated into that of a soccer ball. An icosahedral cluster (as opposed to the truncated form) would be a tougher individual to sell to the world at large.

Then there is the name. Something as extravagantly titled as buckminsterfullerene must surely have an interesting story attached, whereas soccerene or spherene, as it might have been called, would probably now seem trite or prosaic. Buckyballs and buckytubes, for all that *Nature* has avoided the terms, are catchy.

But in terms of illuminating the process by which science is done — a reasonable objective of any book of this sort — the fullerene story has in its favour an abundance of interdisciplinarity. Both books begin in interstellar space and end by roaming the borders of nanotechnology, taking in on the way combustion science, architecture, biological growth and form and solid-state physics.

Those who have publicly or privately deplored fullerene hype should find little to fault here — the authors show why the breadth of fullerene science warrants the excitement, but their assessments are commendably restrained. And it was a matter of some relief to me that both books are relatively free from the swaggering, sound-bite-spewing scientific caricatures that bedevil so much popular science writing.

Perhaps the major attraction of the C_{60} story, however, is that it required a leap of imagination and a generous helping of

intuitive faith. This is what takes it beyond the mundane business of science as cautious, incremental steps forward, of Kuhn's 'normal' science. Would the tale be half so gripping, for example, if a total synthesis of C_{60} had been the means for its mass production? A genuine organic synthesis would have been a far greater intellectual *tour de force* than its formation in an arc reactor (it still would be); but that would also have been a matter of steady, careful and wholly rational steps converging on the final goal, not the consequence of a physicist's "crazy idea". Sadly, perhaps, but for good reason, I cannot see a popular book being written on the total synthesis of taxol.

What in fact happened was an appealing reversal of the usual progression of research. The story began on the high-tech cluster-beam apparatus in Rick Smalley's laboratory at Rice, an instrument worth hundreds of thousands of dollars, and progressed to ever simpler, cheaper

"What made mass production of C_{60} so difficult was disbelief that it could be so easy. Thus fullerene chasing became science on an ever more modest scale"

and more *ad hoc* arrangements, through Wolfgang Krätschmer's arc-discharge system inside a bell jar at Heidelberg to the absurd extreme of Don Bethune's burning-peanut can in a fume cupboard at IBM Almaden. What made mass production of C_{60} so difficult was disbelief that it could be so easy. Thus fullerene chasing became science on an ever more modest scale, to which postgraduate and undergraduate students could make valuable contributions, and in which the financial margin between success and failure could be as little as £80, the cost of replacing a burnt-out transformer.

This was an essential part of the fullerene explosion. Not only was it possible, in late 1990, to produce C_{60} in large quantities; it was also possible to do so very cheaply. Anyone could play. My own first glimpse of the red powder of semi-refined fullerenes came in a laboratory in northeast China in 1992, which had its own makeshift arc reactor. But by the end of 1990 you didn't even need that: C_{60} was available by mail order.

There is also the spice, in these accounts, of watching top-rate scientists adrift in a foreign place. How does soot form? The musings of physical chemists Smalley and Harry Kroto were covered in opprobrium by the combustion community. How does one dissolve C_{60} from soot? Benzene is the screamingly obvious solvent, but not to the Heidelberg physicists. How do you make a closed sphere from hexagons? The impossibility of doing so is

evident from the theorem due to Euler that is taught in high schools, but the discoverers of C_{60} spent frustrating days at the task. None of this is to the shame of the scientists concerned, but is simply an indication that great discoveries need not, and usually do not, require omniscience.

Aldersey-Williams is thorough as far as he goes, but he does not go everywhere. Remarkably, for example, as prominent a part of the fullerene myth as David Jones's prescient article in 1966 on "graphite balloons" gets no mention, nor does the theoretical work on C_{60} by Eiji Osawa or the Soviets D. A. Bochvar and E. G. Gal'perin in the 1970s. And the reader is left to ponder why Orville Chapman at the University of California, Los Angeles, was to be found trying to synthesize the molecule in 1980, five years before its 'discovery'.

Sometimes too the author's grip wavers, particularly in the account of fullerene superconductivity. In characterizing superconductors as poorly understood, he fails to make a distinction between conventional materials and the high- T_c copper oxides. And the fullerene development will no doubt seem all the more technologically significant when the reader is not told that the 77-K barrier was broken long before doped C_{60} started creeping towards it.

On the other hand, Aldersey-Williams takes us on some illuminating detours through the symmetry properties of polyhedra and the strange and recondite world of Buckminster Fuller the architect, many of whose meanderings he dismisses rightly, if irreverently, as exemplary pseudoscience.

Baggott is more comprehensive, but less reflective. He tells the story straight, in more or less chronological fashion with few diversions. A minor drawback of this is that his book will occasionally be tough going for nonscientists, and that it sometimes sags under the weight of extraneous detail. But it provides an overview of the state of the field that is as well informed and as up to date as one could wish.

For production values, Baggott wins hands down: his book is lavishly illustrated, largely with figures from the original sources. But whereas Baggott is closer to his topic, Aldersey-Williams maintains a distance that allows him to tread on more toes, no bad thing in a field laden with dispute and rivalry. So the two books serve slightly different ends, but both are very well written and rewarding. You will get an excellent account of the story from either, and the fact that they have appeared now rather than closer to the 1990 breakthrough means that either can provide a perspective that should persuade the reader that this one will run and run. □

Philip Ball is an associate editor of *Nature*.

How molecular motors work

James A. Spudich

What is the molecular basis of cell movement and changes in cell shape? The integration of three approaches is revealing how the molecular motors that drive these processes move and produce force.

MYOSIN has long been known to be a crucial component of muscle contraction. This motor molecule forms bipolar thick filaments that interdigitate with thinner actin filaments in the sarcomere, forming the basic contractile unit of muscle. Our current concepts of how muscle contracts and other cells move or change shape are based on our understanding of the molecular architecture of the sarcomere^{1,2} and on physiological experiments on muscle fibres begun 25 years ago^{3,4}.

At about the same time, discoveries of myosin in cells other than muscle came largely from studies in model eukaryotic cell systems such as *Physarum*, *Acanthamoeba* and *Dictyostelium*. A conventional, muscle-like myosin that forms bipolar thick filaments exists in these and virtually all other eukaryotic cells. I use the term myosin throughout to refer to this conventional myosin, also called myosin II to distinguish it from other motors of the myosin superfamily discovered more recently^{5,6}. Classical cell-biological approaches have implicated this myosin in cytokinesis and other forms of cell-shape change and movement^{7,8}. Antisense experiments⁹ and myosin gene knockout experiments¹⁰ in the 1980s provided genetic proof that myosin is essential for cytokinesis and change in cell shape during development in *Dictyostelium*, but revealed, surprisingly, that cells can still migrate on surfaces in its absence.

There has been an explosive rate of discovery of new molecular motors in the past decade. This rapid advance stemmed from the discoveries of the myosin I family^{11,12}, kinesin¹³ and cytoplasmic dynein¹⁴. It is likely that a given cell has 50 or more distinct molecules that operate on actin filaments and microtubules to produce the many types of movement cells undergo. Taken together with the ATP-driven motor molecules operating along DNA, the movement of ribosomes along messenger RNA, and motors that have yet to be discovered, the number of molecular motors in a cell is probably closer to 100.

Biochemists have successfully developed *in vitro* assays for the function of their favourite enzymes: in the case of molecular motors, these enzymes move along their respective tracks. I present here a brief, personal perspective of the development of quantitative *in vitro* motility assays for the myosin molecule, and discuss the importance of integrating such assays with molecular genetics and structural biology. I focus on myosin because more is known about this protein than about other molecular motors, although this gap is closing rapidly^{15,16}. I also suggest crucial roles for two apparently non-conserved surface 'loops' on the motor domain of myosin. I will not deal with many important advances using other approaches (see refs 17–21 for reviews), and my account is not intended to be comprehensive.

Actin-activated myosin ATPase cycle

The mechanism of the transduction of the chemical energy derived from ATP hydrolysis to force production and movement by myosin is still not fully understood. But the integration of three approaches—*in vitro* motility assays, molecular genetics and structural biology—has provided considerable support to the mechanochemical actin-activated myosin ATPase cycle^{22,23} shown in Fig. 1. According to this hypothesis, myosin exists in two states while tightly bound to actin: a state before the power

stroke of the globular head domain of myosin (upper left panel) and a post-stroke state (upper right panel). Because the myosin is anchored in a thick filament (not shown) by its α -helical tail (a small portion of which is shown in Fig. 1), the transition from the pre-stroke to the post-stroke state gives rise to about a 10-nm displacement of the actin filament relative to the myosin. Step 1 in Fig. 1 involves the binding of ATP to the globular head domain of the myosin molecule, which results in sufficient conformational change to cause the rapid dissociation of the myosin from the actin. In step 2, the ATP is rapidly hydrolysed to ADP and P_i , which stay tightly bound to the myosin. This myosin $\cdot P_i \cdot ADP$ is in rapid equilibrium with an actin $\cdot$ myosin $\cdot P_i \cdot ADP$ complex, but this is a low-affinity interaction. Step 2 could involve further conformational changes in the myosin, as depicted schematically in the lower panels. This step is followed by a slow transition (step 3) to an activated, high-affinity form of the actin $\cdot$ myosin complex (actin $\cdot$ *myosin $\cdot P_i \cdot ADP$, presumably comprising a conformationally strained form of the myosin now in its pre-stroke state). The total cycle time (t_c) is limited by step 3 and is determined by how fast the low-affinity actin $\cdot$ myosin $\cdot P_i \cdot ADP$ complex is converted to the high-affinity actin $\cdot$ *myosin $\cdot P_i \cdot ADP$. This conversion triggers the release of P_i (step 4)^{22,23} which in turn triggers a putative large conformational change (step 5) that gives rise to a step in motion of about 10 nm. This conformational change allows the ADP to dissociate²⁴. The combination of steps 4 and 5 is referred to as the 'strongly bound-state time' (t_s), as the myosin is strongly bound to the actin throughout these steps. In the case of skeletal muscle myosin, t_s is about 2 ms (ref. 25). Dissociation of ADP allows rapid binding of ATP, and the crossbridge cycle repeats. Closely related models have variations in the later steps in this cycle.

This cycle was developed from detailed kinetic measurements of the steps involving the interactions of myosin with nucleotides and actin, and of the myosin-catalysed hydrolysis of ATP. The next step was to study the ability of the motor to translocate along its track. The establishment of quantitative *in vitro* motility assays for the actin–myosin system^{26,27} not only provided the means to quantify such motor movement *in vitro*, but led to the discovery of the class of motors now called the kinesin family^{13,15,16}.

In vitro motility assays

The early motility assays consisted of myosin-coated beads, which could be shown to undergo ATP-dependent movement along polarized actin cables attached to isolated *Nitella* cell membranes²⁶. Then it was found that similar movement *in vitro* could be obtained with nothing more than highly purified actin, myosin and ATP²⁷, and a simpler myosin-coated surface assay²⁸ was used to show that the myosin head, called subfragment-1 (S1), is all that is needed for ATP-dependent displacements²⁹. This assay was then modified, by attaching an actin filament to a glass needle, to estimate the force developed by S1 (ref. 30).

A feedback-enhanced laser-trap system³¹ has now allowed the observation of definitive single steps of 10–20 nm and forces of up to 7 pN, which almost certainly reflect the productive inter-

action of a single myosin molecule with a single actin filament. In this assay, the position of the trapping laser beam can be rapidly changed by an electronic feedback system to clamp an actin filament being pulled by a single myosin molecule. Using the glass-needle assay at low myosin densities, similar step sizes have been observed: the maximum force measured, although not truly isometric, gave values of ~ 5 pN (ref. 32). An approximately 10-nm stroke size is consistent with the conventional 'swinging crossbridge' model² for contraction (Figs 1 and 2), as the myosin head domain (S1) is itself about 20 nm in length. Very similar stroke sizes for kinesin moving along a microtubule had previously been observed³³, and the mechanism for movement by all molecular motors is likely to be fundamentally the same.

Although these data are most consistent with a tight coupling where one ATP hydrolysis event gives rise to one step in motion, this scheme has not been directly demonstrated. There has been considerable controversy over how far myosin can move along an actin filament as a result of a single ATP hydrolysis. Several experiments suggest that multiple 10-nm steps can occur following the hydrolysis of a single ATP^{34–38}, whereas others are more consistent with the tightly coupled model predicting about 10 nm per ATP hydrolysed^{25,39,40}. All the experiments before the most recent work^{31,32} suffered problems in determining how many myosin molecules were actively involved in the processes being measured, and by the inability to measure accurately the amount of ATP hydrolysed during movement in the *in vitro* motility assays. Small steps produced by single myosin molecules have now been directly measured, but measurement of the amount of ATP hydrolysed in these experiments is still not technically possible.

Molecular genetics

Quantitative assays for ATPase activity, velocity of movement, stroke size and force can be integrated with assays in which molecular motors are genetically altered. *Dictyostelium* has emerged as a powerful system for this purpose. Both cytokinesis and development in *Dictyostelium* exhibit an absolute requirement for myosin, allowing the functional state of mutant myosin molecules to be assessed *in vivo*⁴¹. Further, mutant molecules can be expressed and purified to homogeneity using *Dictyostelium* as the host cell. The purified protein can be studied using the assays described above to correlate the results from the *in vivo* functional tests with *in vitro* studies. These features of the *Dictyostelium* system have allowed the production of many randomly mutated myosin forms which, by *in vivo* testing, group into three categories: fully functional, partially functional and nonfunctional (K. Ruppel and J.A.S., manuscript in preparation). Many of the partially functional and nonfunctional mutant myosins have been purified and characterized as to where they are blocked in the ATPase cycle (see Fig. 1). Mutants altered in the ATP-binding pocket of S1, for example, are blocked in the step that involves dissociation of the tightly bound actin-myosin complex by ATP. Other mutant forms of myosin bind actin and release from actin with ATP, but are blocked in the hydrolysis of ATP to ADP and P_i. Where are these mutations with respect to the nucleotide- and actin-binding domains of the molecule? To answer this question, structural information is needed.

Structural biology

Among the exciting developments of the past few years are the high-resolution X-ray structure determinations of actin⁴², of chicken S1 (ref. 43) and of the regulatory part of the scallop S1 domain⁴⁴. The actin-filament structure has been inferred by model building in comparison to X-ray powder diagrams of pelleted F-actin⁴⁵, and has been fitted into three-dimensional reconstructions from electron micrographs of F-actin⁴⁶. By fitting the S1 crystal structure into the envelope of the actin-S1 complex viewed by electron microscopy, the orientation of the S1 on the actin filament has been determined, and the side chains

on actin interacting with those on the myosin can be inferred^{47,48}. Binding of S1 to actin involves both charge-charge and hydrophobic interactions.

A striking feature of the S1 structure is that the nucleotide-binding site and the actin-binding site, which have to communicate with each other during contraction, are nearly 4 nm apart (Fig. 2). Thus, the region of the protein between the nucleotide-binding and the actin-binding sites must act as a communication zone. The importance of this region is emphasized by the fact that several of the more interesting *Dictyostelium* mutations discussed above lie in this part of the molecule.

Another striking feature of the S1 structure is the presence of a 'lever arm' (depicted schematically in Fig. 2) comprising about 73 amino-acid residues of the carboxy-terminal portion of the S1 heavy chain, in the form of a long, single α -helix. Two calmodulin-like light chains, the essential light chain (ELC) and the regulatory light chain (RLC), are wrapped around this α -helix^{43,44}, which could provide the heavy chain α -helix with sufficient rigidity to act as a lever⁴³. The lever arm is about 10 nm long, so its swing could produce a stroke size close to that measured in the laser-trap experiments³¹.

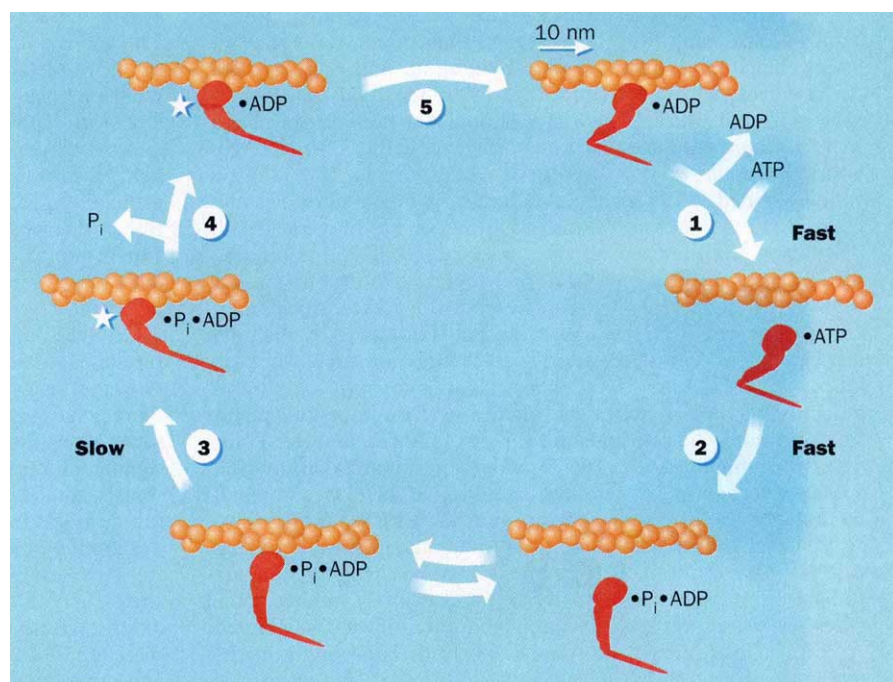
The power of integrating the three approaches discussed above—in *vitro* motility assays, mutagenesis and structural biology—is illustrated by studies of the lever arm. Weakening of the lever arm by removal of one or both light chains causes a 50–90% reduction in the velocity with which that myosin moves *in vitro* without significantly altering the actin-activated ATPase⁴⁹. A truncated version of myosin with a $\sim 50\%$ shorter lever arm prepared using the *Dictyostelium* system⁵⁰ moves actin at about one-half the velocity of the native enzyme in the *in vitro* motility assay, without a corresponding drop in the rate of actin-activated ATP hydrolysis. Deletion of both light-chain binding sites removes essentially all the lever arm, and this truncated *Dictyostelium* myosin moves actin at less than 10% of the velocity of the native enzyme, without a corresponding drop in the actin-activated ATPase⁵¹. These results are predicted by the model described above as a shorter lever arm would give rise to a smaller stroke size, d , for each ATP hydrolysed (see Figs 1, 2), and the velocity of actin in the *in vitro* motility assay in the presence of a saturating number of myosin molecules is given by d/t_s , where t_s is the strongly bound-state time (see Fig. 1 and, for further explanation, see below). The creation of a myosin molecule with a longer lever arm should result in a larger stroke size and therefore a higher velocity of movement than that of the native myosin. This experiment would provide a convincing demonstration of the lever-arm concept.

Critical roles for surface loops

Of considerable interest are two surface loops in the myosin motor domain that are flexible and therefore do not show up in the crystal structure⁴³. Although they are present in skeletal, cardiac and smooth muscle myosins, as well as in myosins from other cells, these loops are very poorly conserved both in length and in amino-acid sequence. Yet they are localized at interesting sites in the structure of the motor. Loop 1 (residues 204–216 in the crystal structure) is near the ATP-binding pocket, and loop 2 (residues 627–646) is at the actin-binding site (Fig. 2). Structural studies indicate that loop 2 interacts with the negatively charged amino-terminal part of actin.

Recent studies of loop 2 (ref. 52) illustrate the importance of the integration of the three approaches discussed here. Chimaeras comprised of the *Dictyostelium* myosin backbone and the *Dictyostelium* loop 2 replaced with that from skeletal muscle made the enzyme 5–6-fold more active as measured by the $V_{\max}$ of its actin-activated ATPase activity. Alpha-cardiac-*Dictyostelium* and β -cardiac-*Dictyostelium* chimaeras are also more active than the native *Dictyostelium* enzyme, but less so than the skeletal-*Dictyostelium* chimaera. In fact, the $V_{\max}$ of the chimaeras corresponds reasonably well with those of the native homologous mammalian myosins from which the loop sequences were

FIG. 1 Myosin consists of two heads and a long α -helical coiled-coil tail. For clarity, only one head (S1, the motor domain of myosin) and a short segment of the α -helical tail are shown. The actin·myosin· P_i ·ADP state is shown in the left panel. The figure shows conformational changes at each step leading up to the pre-stroke state shown in the upper left panel, but it is not known at which of the steps the major conformational change occurs.



derived. Thus, this surface loop 'tunes' the rate-limiting step in the actin-activated ATPase cycle, which could be the conversion of actin·myosin· P_i ·ADP to actin·*myosin· P_i ·ADP. Because loop 2 is at the actin-binding site, it seems plausible that it may be directly involved in triggering this conversion, which in turn then triggers P_i release (Fig. 1).

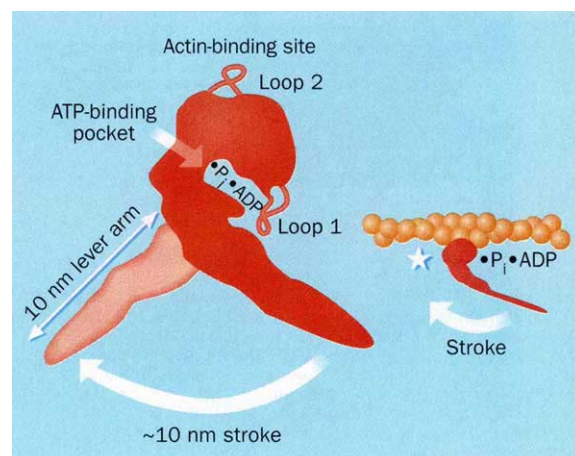
Although the V_{max} values for the actin-activated ATPase activities of the various chimaeras differ by as much as a factor of nearly six, their velocities in the *in vitro* motility assay are the same within a factor of two. How can this be? The answer to this question may be at the heart of how molecular motors work. There are two critical rate constants that must be considered. The first sets the V_{max} for the actin-activated ATPase cycle, for example, the rate of conversion of actin·myosin· P_i ·ADP to actin·*myosin· P_i ·ADP. Under normal conditions where there are many myosin molecules involved in moving an actin filament, a second rate constant, probably the ADP release rate, sets the limit for the velocity of the movement.

Consider movement along a single myosin head. The velocity and ATPase activity are intimately linked as a single head can move the actin filament only at a velocity given by d/t_c , where

d is the stroke size and t_c is the total cycle time of the actin-activated ATPase cycle (see Figs 1 and 2; d is ~ 10 nm and t_c is of the order of 50 ms for skeletal muscle myosin, and therefore the velocity along a single head would be about $0.2 \mu\text{m s}^{-1}$). Two myosin molecules working asynchronously, however, can produce twice the velocity because one head is getting ready while the other is stroking during the strongly bound-state time. This increase in velocity as more heads are brought into play continues until some limit, and that limit is set by the time in which the strongly bound state, t_s , continues. The actin filament cannot move any faster than the heads can let go, and therefore the maximum velocity, V_o , is d/t_s (t_s is ~ 2 ms, so V_o is about $5 \mu\text{m s}^{-1}$ for skeletal myosin). So what sets t_s ?

Consider the following plausible sequence of events: the total cycle time is determined by how fast the weakly bound actin·myosin· P_i ·ADP complex is converted to the strongly bound actin·*myosin· P_i ·ADP, which is largely set by loop 2 (see Fig. 2); the formation of actin·*myosin· P_i ·ADP triggers the release of P_i , which in turn triggers the conformational change producing the ~ 10 -nm stroke; this conformational change is followed by ADP release. ADP dissociation is essential

FIG. 2 The panel on the right depicts the actin·*myosin· P_i ·ADP state of Fig. 1, left. Small curved arrow, direction of the conformational change resulting in a stroke of about 10 nm. This stroke of the lever arm of S1 pulls on the myosin tail, which is attached to the myosin thick filament (not shown) and causes a relative movement of the myosin filament and the actin filament. The blow-up on the left shows a highly schematic view of just the S1 (head portion) of the myosin molecule. The position of the two loops that may set the critical rate constants for the V_{max} of the ATPase (loop 2) and the maximum velocity of movement of the motor (loop 1) are shown.



for the binding of ATP, which dissociates the strongly bound actin-myosin complex. Note that t_s is independent of t_c and is set by the chemistry of the nucleotide-binding site and its immediate environment, which determines how fast the ADP releases so ATP can bind. In other words, although the conversion of the weakly bound complex, actin·myosin·P_i·ADP, to the strongly bound complex, actin·*myosin·P_i·ADP, limits $V_{\max}$ of the actin-activated ATPase, the ADP release rate determines the value of t_s and therefore the maximum velocity of movement^{24,53}.

By changing the chemistry at or near the nucleotide binding site, the rate of release of ADP and therefore of the maximum velocity of movement, V_o , can be controlled. The nucleotide-binding site itself is highly conserved but is bordered by the only other apparently non-conserved surface loop in the S1 structure, loop 1 (Fig. 2). Like loop 2, loop 1 varies in both length and sequence between myosins, and there is a strong possibility that this loop tunes the rate constant for the ADP release rate. Thus, making chimaeras of the *Dictyostelium* and mammalian myosin species where loop 1 is replaced should give the opposite result to that obtained by manipulating loop 2. That is, those chimaeras will change in velocity but not in the rate of actin-activated ATPase. This is not likely to be an all-or-none effect, as changing the myosin structure in virtually any way is likely to have some effect on both the velocity and the rate of actin-activated ATPase. Nevertheless, the trend is likely to be strongly in the direction predicted. In the natural evolution of the myosin-II family, these two loops may have been altered largely in parallel to keep the percentage of the cycle time (t_c) that myosin heads are in their strongly bound state (t_s) constant⁵⁴. This ratio t_s/t_c is often referred to as the duty ratio.

The potential importance of these two surface loops on myosin are supported by various observations. For example, there are several cases where alternative splicing gives rise to isoforms of myosin that are different only in one or the other of the loops (refs 55, 56; R. S. Adelstein, personal communication). Further, a cold-sensitive myosin mutation that arose after random mutagenesis of the *Dictyostelium* genome is a missense mutation in loop 2 (B. Patterson and J.A.S., in preparation). The concept that apparently non-conserved surface loops serve as critical determinants in tuning critical rate constants, often for nucleotide product release, appears to apply to a multitude of other enzymes and may indeed be a common theme in protein structure and function.

Conclusions and future perspectives

The ability to measure displacements and forces with single motor molecules opens a new era for exploring how these molecules use the energy of ATP hydrolysis to perform mechanical work. Further, laser-trap technology allows many types of measurements that could be applied to virtually any protein. For example, the elasticity of a protein molecule can now be measured directly, and conformational changes associated with transitions between states are within reach of being explored. For motor proteins, the integration of these approaches with molecular genetics and structural analyses is enormously powerful.

Although myosin is the best-studied molecular motor, microtubule-based motors offer special opportunities to understand how motors work. In part because of its relatively small size, the mechanism of action of kinesin is rapidly approaching the same level of understanding as myosin, and incorporates two significant new features. First, this motor works processively; that is, unlike myosin, a single molecule moves along a microtubule track for many seconds before dissociating. Second, the thermodynamic and kinetic properties of kinesin are different from those of the myosin molecule⁵⁷⁻⁵⁹; these differences must correlate with the different behaviours of these motors and their different functions *in vivo*. Finally, within the kinesin family there are motors that move in one direction along a microtubule and others that move in the opposite direction. Is this directionality switch due to a change in the thermodynamics of the kinesin-microtubule interaction, or is it a result of a structural difference in position of a putative lever arm? Microtubule-based motors also offer the opportunity to examine the generality of the proposed mechanistic features of the myosin family. That is, what is the nature of the communication zone between the microtubule-binding site and the nucleotide-binding site? Is there an equivalent of the myosin lever arm in the structure of the microtubule-based motors? Are there loops in those motors that are involved in tuning the critical rate constants that determine the $V_{\max}$ of the ATPase and the maximum velocity of movement, as proposed here for myosin? Crystal structures of kinesins will be necessary to answer these questions. □

James A. Spudich is in the Departments of Biochemistry and Developmental Biology, Stanford University School of Medicine, Stanford, California 94305, USA.

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Long-term synapse loss induced by focal blockade of postsynaptic receptors

Rita J. Balice-Gordon* & Jeff W. Lichtman†

Department of Anatomy and Neurobiology, Washington University School of Medicine, 660 South Euclid Avenue, Box 8108, St Louis Missouri 63110, USA

Focal application *in vivo* of α -bungarotoxin to block neurotransmission in a small region of a neuromuscular junction causes long-lasting synapse elimination at that site. In contrast, blockade of neurotransmission throughout a junction does not cause synapse elimination. These and related experiments indicate that active synaptic sites can destabilize inactive synapses in their vicinity.

THE persistence of memories in humans over many decades argues that experience can cause long-lasting structural modifications in synaptic circuitry. How such modifications occur is poorly understood, partly because of the difficulty of monitoring synaptic connections in the central nervous system over long time periods. Long-term monitoring of synaptic connections is possible however, at the neuromuscular junction, a simple and accessible synapse where structural changes in connections (synaptic plasticity) have been directly observed¹⁻⁷.

One well known instance of plasticity at neuromuscular junctions occurs in neonatal life when each junction undergoes a transition from innervation by multiple motor axons to innervation by only one^{8,9}. The loss of synapses that underlies this change in connectivity is a protracted process spanning several weeks. In mice, the losing axon at each junction gradually relinquishes synaptic territory, finally withdrawing when all of its synaptic endings have been removed¹⁰. The removal of presynaptic terminals is matched by a corresponding loss of postsynaptic acetylcholine receptors (AChRs) from the muscle fibre membrane at the same sites^{2,11}. The changes in AChR density in the postsynaptic membrane begin before the overlying nerve terminals are removed, suggesting that the postsynaptic cell may be instigating the removal of some of the synapses impinging on it while at the same time maintaining the rest.

One way a developing muscle fibre might maintain some AChR clusters (at sites occupied by one axon) while simultaneously disassembling nearby AChR clusters (at sites occupied by other axons) would be for it to sense differences in the timing of neural activity from the different axons. In particular, if the activity pattern of the set of synaptic terminals derived from one axon (a cartel¹²) was different from the activity pattern of the synapses derived from another axon, then all the receptor regions within a multiply innervated junction would not be synchronously activated. In contrast, once a muscle fibre was innervated by only one axon then that cartel would activate all the remaining AChR regions synchronously. If asynchrony of receptor activation leads to synapse elimination, then we hypothesized that it should be possible to perturb synaptic maintenance even at singly innervated junctions by desynchronizing AChR activation at different sites within a junction.

Changes induced by focal AChR blockade

AChR activation within a junction was desynchronized by focally blocking neuromuscular transmission with α -bungarotoxin (α btx), an irreversible ligand of the AChR which prevents

receptor activation by acetylcholine. This was accomplished in the sternomastoid muscle of living adult mice by applying many pulses of positive pressure to a micropipette filled with either fluorescently labelled or unlabelled α btx in saline positioned over one region of a superficial neuromuscular junction under visual control (Fig. 1). In most experiments the same site was saturated with α btx two times 48–72 h apart.

At many neuromuscular junctions, focal blockade of neuromuscular transmission elicited pre- and postsynaptic changes beginning several days after the application of α btx. The principal change was a gradual and progressive elimination of synaptic sites that were blocked. Figure 2 shows an example of a junction in which about 20% of the AChRs throughout the junction were labelled with rhodamine conjugated α btx ($R\alpha$ btx) on day 0 (a dose that is unlikely to interfere with effective neuromuscular transmission¹³) and the bottom region was then twice saturated with unlabelled α btx (day 0 and day 3). Signs of AChR loss were evident three days after the second application of unlabelled α btx (Fig. 2, bottom row, day 6). Over the subsequent 5 days, progressively more of the previously stained AChRs in the blocked area disappeared (Fig. 2, bottom row, days 6–11) showing that AChRs inserted before the blockade were lost from that region. At day 31, additional $R\alpha$ btx was bath applied to the junction to label AChRs inserted since the time of the first focal blockade (little of the original $R\alpha$ btx staining was left anywhere because of turnover of AChRs). Although the additional $R\alpha$ btx stained the top of the junction at day 31 no new staining was evident in the region that was previously blocked. Thus, AChR molecules already in the postsynaptic membrane selectively disappeared from blocked sites (by either migration away or internalization) faster than turnover caused receptors to be removed from unblocked parts of the junction. In addition, the blocked region of the junction either did not incorporate any newly synthesized AChRs into its membrane or did not maintain a detectable number of the AChRs that were incorporated there.

The other major change we observed at the sites of focal blockade was loss of presynaptic nerve terminal staining. Using 4-Di-2-Asp to stain motor nerve terminal mitochondria^{1,2,11}, we observed loss of brightly stained presynaptic elements from the area overlying the region of AChR blockade. The loss of nerve terminal staining induced by focal blockade progressed over the same interval as loss of AChR staining (Fig. 2, top row). The protracted nature of these changes suggests that focal blockade sets into motion a relatively slow process that continues until the blocked region is eliminated. The fact that no nerve terminal sprouts or AChRs reappeared at the previously blocked sites one month later suggests that the changes induced by focal blockade are permanent. Permanent loss of pre- and postsynaptic staining also occurs in other situations in which synapse elimination takes place^{2,11,14,15}.

* Present address: Department of Biology, David Mahoney Institute for Neurological Sciences, University of Pennsylvania, 415 South University Avenue, Leidy Room 319, Philadelphia, Pennsylvania 19104-6018, USA.

† To whom correspondence should be addressed.

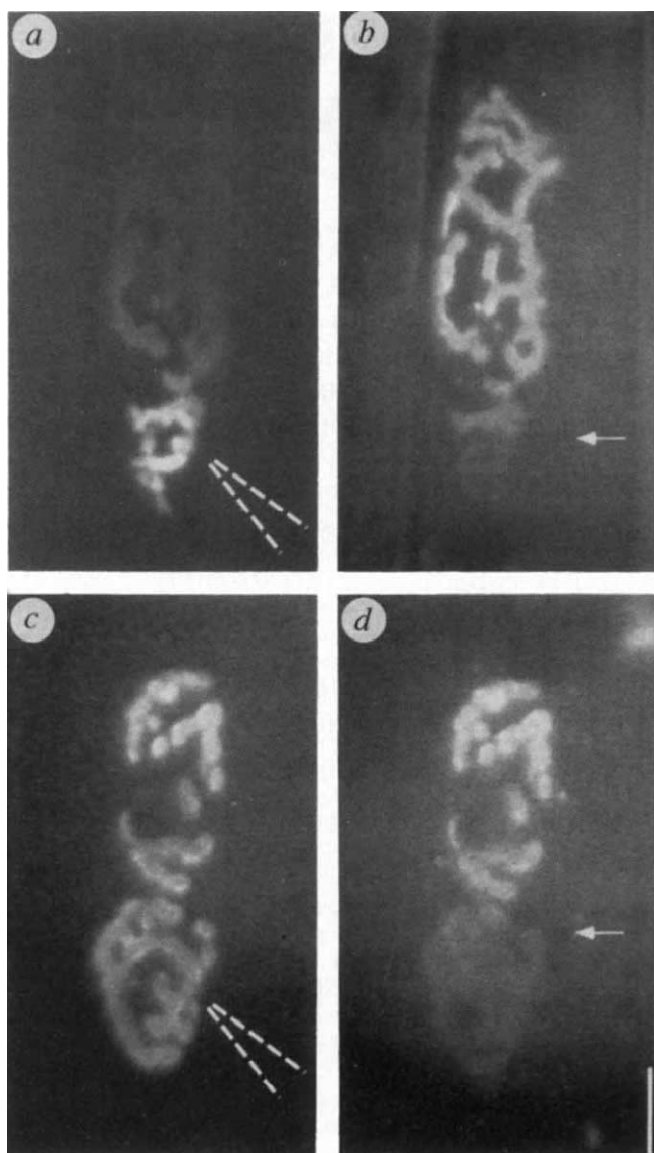


FIG. 1 Focal application of α -bungarotoxin to regions of adult neuromuscular junctions saturates AChRs. Adult mice (25–25 g) were anaesthetized and the sternomastoid muscle exposed as previously described¹. **a**, A microelectrode, pulled to have a sharply tapering shank typical of an intracellular electrode with a blunt end typical of a patch electrode (tip diameter, $<1\ \mu\text{m}$, resistance 7–10 mOhms measured in 3M KCl) was filled with a mixture of 20–25 $\mu\text{g ml}^{-1}$ rhodamine conjugated αbtX ($\text{R}\alpha\text{btX}^{1,2,11}$), 25–50 $\mu\text{g ml}^{-1}$ unlabelled αbtX (BioToxins, Inc.) and 10 $\mu\text{g ml}^{-1}$ sulphorhodamine 101 (Molecular Probes) as a fluorescent tracer in lactated Ringer's (Baxter). The electrode (dashed lines) was positioned near a junction under epillumination using a polarizing filter cube and long working distance $\times 40$ water immersion objective (Zeiss, 0.75 n.a.). Perfusion of Ringer's (10 ml min^{-1}) across the surface of the muscle was adjusted so that the stream from the electrode was directed toward the area of interest and away from the rest of the junction (see Fig. 2, bottom row, 'during puffing'). Small quantities of the αbtX solution were then released from the tip of the electrode using pressure pulses (10–20 p.s.i.) delivered at 5–20 Hz in trains of 50–500 ms duration for 5–10 min. **b**, In control experiments, the degree of saturation was determined by subsequently applying Lucifer yellow conjugated αbtX ($\text{LY}\alpha\text{btX}$, Molecular Probes) and noting the presence or absence of $\text{LY}\alpha\text{btX}$ binding in the region of the junction near the electrode (region below arrow). Motor nerve terminals were subsequently stained with a vital, non-toxic mitochondrial dye, 4-Di-2-Asp^{1,2,11} to determine whether or not the overlying motor nerve terminals were damaged during the application of αbtX (not shown but see Fig. 2). Digital images of neuromuscular junctions were obtained using a low-light SIT video camera and an image processor as described previously^{1,2,11}. Two to three days later, the entire procedure was repeated. Junctions were then followed over the next 1 to 8 weeks at intervals ranging from 2 days to 2 weeks. **c**, To prevent the possibility of photodamage to $\text{R}\alpha\text{btX}$ saturated AChRs having artefactually induced synapse loss, in some experiments (10 junctions in 5 mice) focal blockade was accomplished with unlabelled αbtX . Neuromuscular junctions were briefly stained (1–2 min) with $\text{R}\alpha\text{btX}$ so that less than 20% of the postsynaptic AChRs were labelled. In this way the postsynaptic distribution of AChRs could be visualized while leaving synaptic transmission unaffected. A microelectrode (dashed lines) filled with unlabelled αbtX (100 $\mu\text{g ml}^{-1}$) in Ringer's solution was positioned near a junction and small quantities of αbtX released with pressure as described above. **d**, In control experiments, saturation was checked with a subsequent application of $\text{LY}\alpha\text{btX}$. The parameters, in particular the number of puffs, necessary for saturating a junctional area were determined from this kind of control experiment. In a few experimental junctions, a low dose of $\text{LY}\alpha\text{btX}$ was applied after puffing, as in the control experiments, to confirm the blockade without saturating the rest of the junction. In all figures digital images of multiple focal planes were aligned and out of focus blur was removed using previously described computational methods¹⁰. Scale bar, 20 μm .

Of 55 junctions (in 23 mice) receiving focal αbtX to the same site twice, 19 (~35%) showed loss of pre- and postsynaptic staining comparable to that shown in Fig. 2. Thirteen (~24%) showed no evidence of pre- and postsynaptic elimination and 23 (~42%) showed changes that indicated they were inadvertently damaged by the procedure, resulting in denervation (absence of 4-Di-2-Asp staining) and/or muscle fibre degeneration (presence of cellular debris and absence of striations). Junctions which showed no evidence of loss had on average larger blocked areas than the junctions which did change (see below). Focal blockade was also less successful at inducing synaptic changes when junctions were blocked only once. Of 65 junctions in 30 mice in which αbtX was applied only one time, 12 (~18%) showed loss of pre- and postsynaptic staining, 22 (~34%) showed no evidence of pre- or postsynaptic change and 31 (~48%) were damaged by the procedure or not relocated.

Two observations indicated that the loss of presynaptic staining observed with the mitochondrial stain 4-Di-2-Asp was due to the frank withdrawal of motor nerve terminals from blocked sites rather than a redistribution of mitochondria within the nerve terminal. Use of a fluorescent dye that intercalates into nerve terminal membrane (RH 795; ref. 2) showed that regions that had lost 4-Di-2-Asp staining could no longer be stained with RH 795 (6/6 junctions in 3 mice).

In addition, a change in synaptic basal lamina staining indicated structural withdrawal of nerve terminals overlying blocked postsynaptic sites. Acetylcholinesterase and the lectin VVA¹⁶ that stains it are distributed in a 'railroad-track' pattern at normal junctions and fills in to become a single broad band (presumably due to increased access) after nerve terminals have withdrawn^{11,14,17}. At sites that were focally blocked and lost pre- and postsynaptic staining, VVA filled in (4/4 junctions from 2 mice; Fig. 3).

We studied the temporal relationship between nerve terminal withdrawal and AChR loss at sites of focal blockade. In eight junctions that showed evidence of changes 2–3 days after focal blockade, we continued to make observations at 2–3 day intervals during the process of synapse elimination. In each of these junctions, we found time points at which AChR staining was diminished in the blocked sites whereas nerve terminal staining was still present (Fig. 4). In none of these junctions did we see the opposite: nerve terminal staining missing at sites that had normal $\text{R}\alpha\text{btX}$ staining. At most blocked sites we observed either that nerve terminal staining and the AChR staining had already both been lost, or that both pre- and postsynaptic staining still appeared normal. From these results we conclude that nerve terminal withdrawal lags slightly the onset of AChR loss from blocked sites. Thus, the timing of pre- and postsynaptic loss

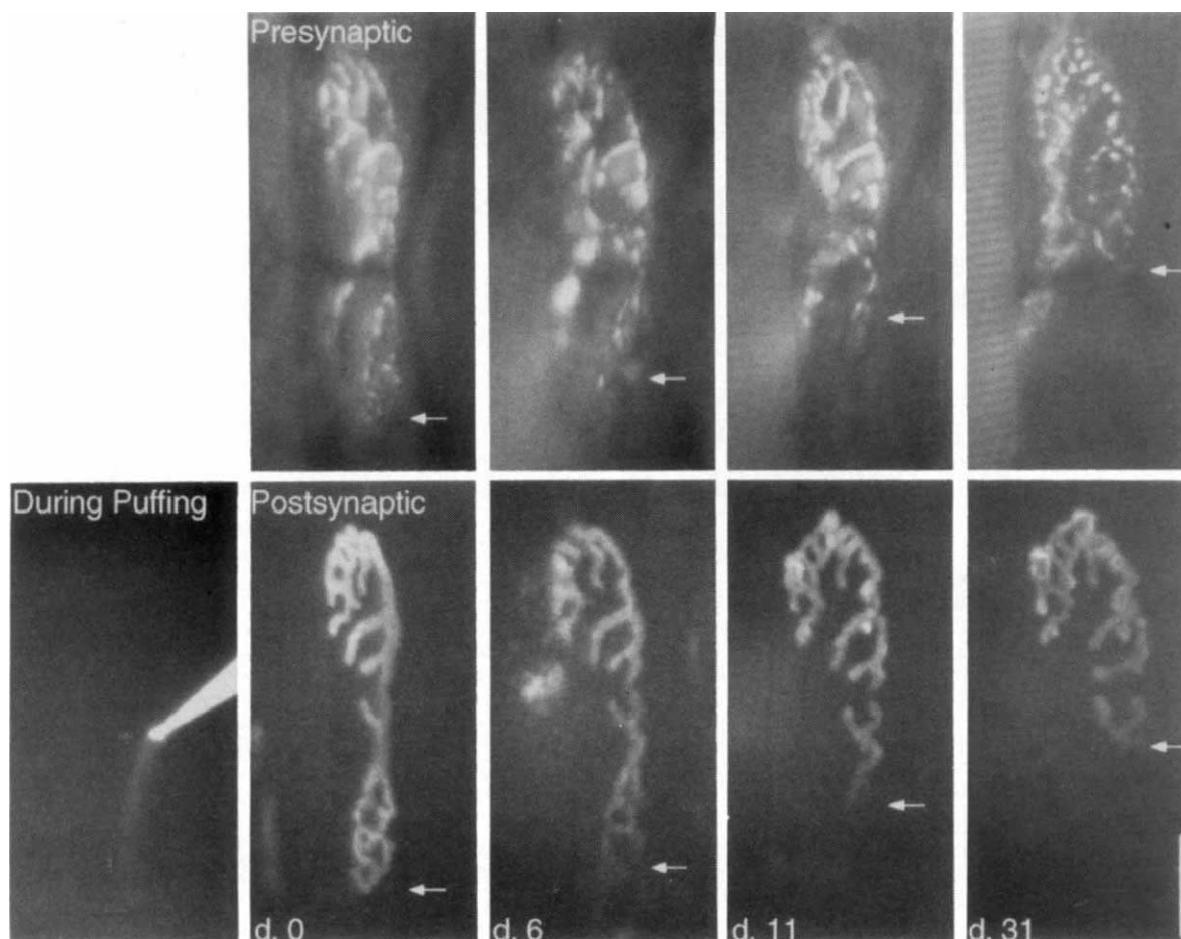


FIG. 2 Gradual loss of presynaptic motor nerve terminal and postsynaptic AChR staining from synaptic regions saturated with α btx. Following staining of about 20% of the AChRs throughout the junction with α btx and saturating all the AChRs in the bottom region of this neuromuscular junction with unlabelled α btx (panel labelled 'During Puffing'), presynaptic motor nerve terminals (top row) and postsynaptic AChRs already in the membrane (day 0–11, bottom row) were followed over time. Immediately following application of α btx (day 0), motor nerve terminal staining was present over the synaptic regions containing saturated AChRs. Over the next several days to weeks, there was a progressive loss of motor nerve terminal staining as well as of the preexisting AChRs (arrows). Moreover, the loss of these areas appeared to be permanent because 31 days later no staining was observed in the previously saturated region of the junction, either using 4-Di-2-Asp (top) or when for the first time additional α btx was applied (bottom). At the last view the remaining AChR-rich areas seem somewhat enlarged perhaps as compensation for the lost sites. The gradual and permanent nature of synaptic change induced by focal blockade of synaptic transmission in

one region of an adult neuromuscular junction is similar to that observed during naturally occurring synapse elimination during development². Scale bar, 20 μ m. We did a variety of control experiments which ruled out the possibility that the changes observed were artefactual. We attempted to damage nerve terminals locally by touching a small region of the nerve terminal with a puffing pipette lacking α btx (24 junctions in 7 mice). In 11 junctions no change was observed; in the 13 junctions in which changes were observed, these consisted of denervation and subsequent reinnervation of the entire junction (4 junctions); and in the remainder, denervation and muscle fibre degeneration and regeneration was observed (9 junctions). In an additional 6 junctions in 3 mice the same solution minus α btx was focally applied twice according to the protocol described above without damaging the junction. In none of these cases did we observe motor nerve terminal or AChR loss, indicating that α btx in the pipette was necessary to induce synaptic changes. Moreover, focal application of unlabelled α btx was as effective as fluorescently tagged α btx, indicating that local phototoxicity could not have caused elimination of the blocked sites.

induced by focal blockade is analogous to that which occurs during synaptic competition between different axons during development and reinnervation^{2,11}.

Blockade throughout a junction

To evaluate the possibility that the changes we observed were caused by the puffing technique itself rather than focal blockade of neurotransmission, we did a variety of control experiments each with the aim of inducing synaptic loss without focally blocking neuromuscular transmission. In none of these experiments could we replicate the effects of focal blockade (Fig. 2 legend).

Saturating the entire junction with two 30-min bath applications of rhodamine or unlabelled α btx 48–72 h apart in the same solution used in the puffing pipette also caused no sign of pre- or postsynaptic loss in any of the junctions studied (22 junctions in 6 mice; Fig. 5). The inability of a complete blockade of neuro-

muscular transmission to cause synapse elimination indicated that α btx itself was not causing synapse loss through, for example, an unexpected impurity that is toxic to synaptic sites. In addition, however, the inability of widespread junctional blockade to elicit synaptic loss implied that neuromuscular blockade is not itself sufficient to cause the changes we observed. Rather, these results suggest that inactive junctional regions are eliminated only in the presence of ongoing activity elsewhere in the junction.

To study this possibility further we examined 50 junctions in which the focal application of fluorescently labelled α btx (to show the size of the blocked region) or a subsequent brief application of Lucifer yellow α btx (to counterstain the blocked area) allowed us to measure the size of the region labelled by puffing. These partially blocked junctions were divided into two groups: those that showed loss and those that did not. The per cent of the junctional area blocked for each group is shown in Fig. 6.

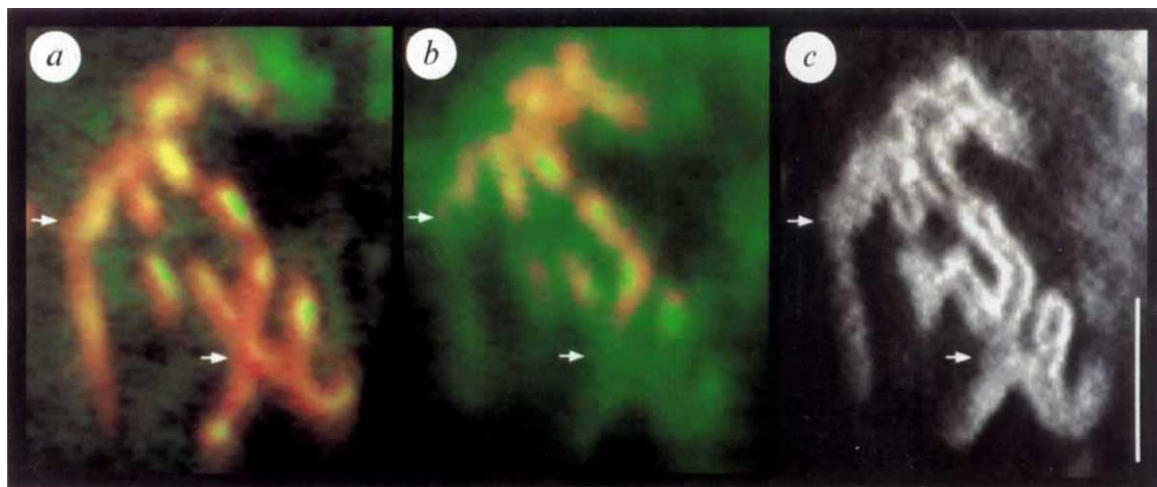


FIG. 3 Evidence that presynaptic motor nerve terminals withdraw from postsynaptic areas saturated with α -btx. *a*, Shown is a portion of a neuromuscular junction saturated twice with α -btx. Immediately following application of α -btx, motor nerve terminal staining with 4-Di-2-Asp (green) was observed over the region of the junction containing saturated AChRs (red). At sites of overlap the juxtaposition of green and red appears yellow. *b*, 21 days later, motor nerve terminals were again stained with 4-Di-2-Asp and AChRs were re-stained with α -btx (red). Former pre- and postsynaptic sites were no longer stained in the region that was previously blocked. *c*, At the time of the second view (same time point as in *b*), the synaptic basal lamina was stained with FITC-

conjugated lectin, VVA, $10 \mu\text{g ml}^{-1}$, ref. 16). The 'railroad-track' appearance of VVA staining is visible in regions of the junction in which motor nerve terminal staining is still present (compare areas above arrows in *b* and *c*), probably because the nerve terminal retards diffusion of the lectin into the synaptic cleft. Where no motor nerve terminal staining remains (areas below arrows in *b* and *c*), the VVA staining has been filled in, typical of areas that have lost motor nerve terminals¹¹. Thus, loss of motor nerve terminal staining is due to frank structural withdrawal of motor nerve terminals from blocked regions of the junction. Scale bar, $20 \mu\text{m}$.

Junctions in which synapse loss was observed had on average smaller blocked regions than junctions that showed no loss (Fig. 6*a*). The probability of loss decreased as the size of the blocked region increased (Fig. 6*b*) indicating that blocked sites were only lost when a substantial area of the junction remained unblocked.

In another series of experiments, we denervated the sternomastoid muscle to eliminate all neuromuscular transmission and then focally blocked regions of neuromuscular junctions (8 junctions in 3 mice). AChR loss was not observed in any of these junctions. Thus non-focal blockade, blockade of large postsynaptic regions and focal blockade in the absence of the nerve were all ineffective in causing synaptic changes. These results strongly suggest that ongoing activity in the remaining parts of the junction is necessary to induce elimination of the blocked regions.

Discussion

The experiments of Hubel and Weisel on the segregation of ocular dominance columns during early postnatal life underscored the importance of the relative activity of competing inputs in determining the final pattern of synaptic connections in the nervous system. Whereas patching one eye profoundly diminished the ability of the inactive eye to maintain synaptic connections in visual cortex, patching both eyes had a far less dramatic effect^{18,19}. This work made a strong case for the idea that differences in neural activity between the eyes, rather than the overall

amount of activity, determine the outcome of the competition between the two eyes for cortical space. These landmark experiments firmly established the relationship between neural activity and long-term synaptic modifications that occur in the nervous system as a consequence of experience.

The experiments we report here seem to show an analogous competitive process between effective and ineffective synapses on individual postsynaptic cells. Blocking activity at all synaptic sites within a neuromuscular junction has far less of an effect than blocking a portion of the sites. Thus the context within which a synaptic site is inactive is critically important to its fate. This work also shows that in certain circumstances synaptic connections in adult animals can undergo the kind of permanent changes that is the hallmark of activity-mediated plasticity during critical periods in development.

Ordinarily in adulthood, the stage at which these experiments were done, all the presynaptic terminals at a neuromuscular junction originate from the same axon and are thus a synchronously active cartel¹² whose terminals normally coexist stably for very long times^{20,22}. If, however, an adult neuromuscular junction is innervated by more than one axon, as occurs following reinnervation¹¹ or following muscle fibre regeneration²³, then synapse elimination ensues until only one cartel remains. The experiments reported here suggest that if the activity patterns of each synaptic cartel multiply innervating a junction were different, then that difference alone might be sufficient to cause syn-

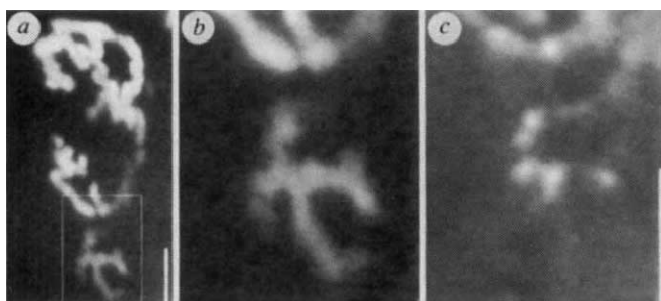


FIG. 4 Loss of postsynaptic AChRs occurs before withdrawal of overlying motor nerve terminals. *a*, Shown is a neuromuscular junction in which the bottom area was saturated 17 days earlier with α -btx. At this view, additional α -btx was bath applied to the muscle to reveal the remaining synaptic regions. The bottom region of this junction is in the process of being eliminated, as indicated by the decreased brightness of α -btx staining in the bottom and along the right branch in this area. *b*, Higher magnification view of the boxed region of junction shown in *a*. *c*, 4-Di-2-Asp staining of the motor nerve terminal in the region in *b* shows partial occupation of faintly stained AChRs. Some faintly staining AChR regions are still occupied by nerve terminals indicating a change in postsynaptic AChR density before nerve terminal withdrawal. Scale bar, $20 \mu\text{m}$.

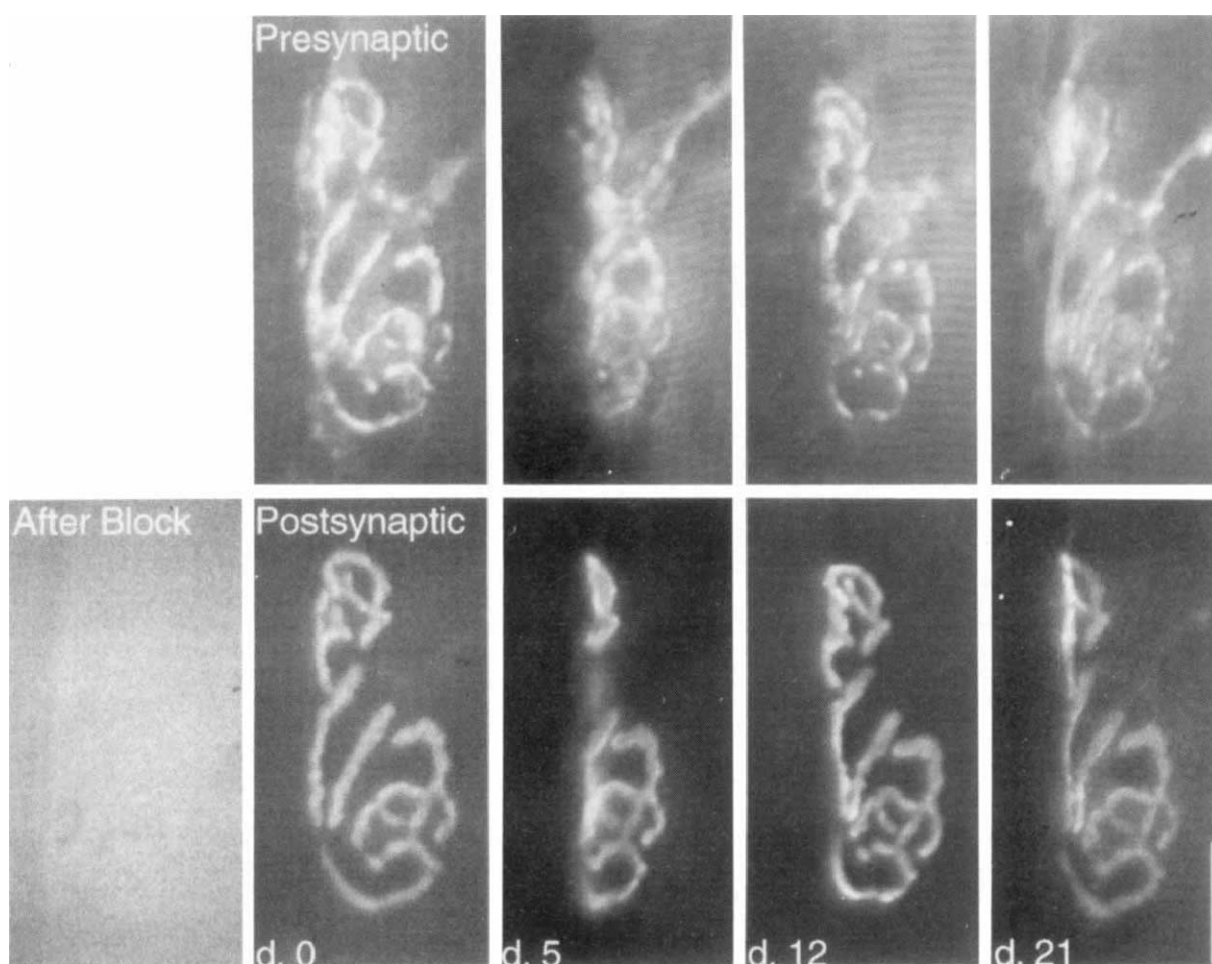


FIG. 5 Blocking postsynaptic AChRs throughout a junction does not result in loss of pre- or postsynaptic sites. Following labelling of about 10% of the postsynaptic AChRs with R α btx (day 0), unlabelled α btx was applied to junctions until all of the remaining AChRs were saturated. Saturation was confirmed by subsequently applying LY α btx to the junction; no staining was observed (panel labelled 'After Block'). AChRs were saturated again 2 days later (not shown). Presynaptic motor nerve terminals (top) and postsynaptic AChRs (bottom) were then followed over 21 days. No loss of pre- or postsynaptic sites was observed. The junction appears thinner at day 5 probably due to slight rotation of the muscle fibre at this view. These results show that the blockade of

AChRs with α btx itself does not induce synaptic loss. Following complete blockade the percentage of blocked AChRs gradually declines because of the receptor turnover⁴⁵. Because nerve terminal loss was not observed at any time following blockade, this experiment shows that there is no threshold level of blockade that causes synapse elimination. Thus, block of 100% of the AChRs in 30% (or less) of the junction (see Fig. 6) induces loss, whereas blocking 30% of the AChRs in 100% of the junction is ineffective. Thus, asymmetry in the efficacy of neuromuscular transmission among the different synaptic sites within a neuromuscular junction is necessary to induce loss. Scale bar, 20 μ m.

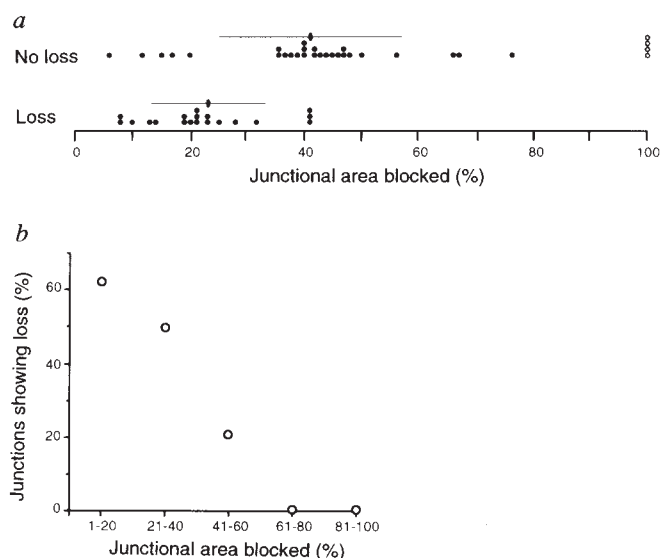
apse elimination. In adult animals, in fact, motor axons projecting to the same muscle typically are not synchronously active but are recruited in a fixed order^{34,25}. One consequence of this is that on multiply (but not on singly) innervated muscle fibres, some sites will be active while others are inactive.

The role of activity in synapse elimination at the neuromuscular junction is complex and controversial. Although some experiments suggest that active axons have a competitive advantage over inactive axons²⁶, others support the opposite conclusion²⁷ and many other experiments suggest a modulating role of activity on the rate of the elimination process (see for example refs 28–31). The present experiments show that a small inactive site is eliminated when pitted against a large active site, but that a smaller active site is neither eliminated by, nor able to eliminate an inactivated region. This argues that in muscle, first, only a threshold amount of postsynaptic activation can destabilize inactive sites and second that an inactive postsynaptic cell may not be able to cause weakening of an active synapse (but see ref. 32).

Previous studies raised the possibility of an important mediating role for the muscle fibre in synapse elimination because decreases in the density of postsynaptic AChRs began before

loss of presynaptic terminals^{2,11}. The same sequence was seen in the present experiments. Although these results suggest that changes in a postsynaptic site could initiate presynaptic terminal withdrawal, they are also consistent with the possibility that nerve terminals instigate their own demise by causing local AChR disappearance and their own disassembly. The present experiments, however, show that a change in the postsynaptic cell (focal blockade) is sufficient to cause normal innervating nerve terminals to withdraw. This strongly supports the idea that the postsynaptic cell plays an important part in maintaining, or choosing not to maintain, the synaptic connections impinging on it implicating retrograde signals in the synapse elimination process. Activity-mediated signals emanating from muscle fibres may also affect pre-synaptic efficacy^{33,34}.

The molecular cascade that underlies synapse elimination is unknown. Local differences in the timing of activation of postsynaptic AChRs presumably lead to local differences in the stability of receptors in the postsynaptic membrane. Receptor disappearance could occur because the AChRs migrate away. The 43K protein that anchors AChRs in the postsynaptic membrane^{35,36} disappears from sites undergoing synapse elimination at the same rate as AChRs themselves³⁷. AChRs could



also migrate by virtue of electrophoresis of membrane proteins as a consequence of voltage gradients induced by activity^{38,40}. Alternatively, a change in the availability of nerve-derived AChR clustering or maintenance factors^{41,42} or their receptors^{43,44} may cause the local disappearance of AChRs within a junction.

AChR clusters could also disappear if the internalization of AChRs is accelerated at inactive sites. Increased turnover (removal and addition) of AChRs occurs in muscles that are inactive⁴⁵. However, inactivity itself does not cause postsynaptic sites to disappear (Fig. 5, see also ref. 11). Rather, AChR activation at one site would have to initiate selective internalization of AChRs at nearby inactive sites. Such a view was essentially posited by Stent in a theoretical treatment of Hebbian mechanisms underlying activity-dependent synaptic plasticity⁴⁶. He proposed that postsynaptic action potentials could cause the internalization of inactive receptors. Consistent with this, tonic muscle fibres which do not fire action potentials do not undergo synapse elimination⁴⁷.

It seems unlikely that the activity of each individual AChR determines whether it is maintained or eliminated. In the blocked region all AChRs disappear including any unblocked AChRs (such as those inserted after the blockade is completed). Similarly with a low dose of α btx some fraction of AChRs are blocked outside the focally blocked area and these AChRs per-

FIG. 6 Probability of synaptic loss is related to the percentage of junctional area focally blocked. *a*, Fifty focally blocked junctions (single and double application) in which we were able to assay the area of the blocked region were separated into two groups, those which did not undergo synaptic loss (No Loss) and those which did (Loss). Although the receptor labelling was usually in a gradient, we could in each junction discern a region of similar intensity (the area of saturation). The percentage of junctional area which was saturated was plotted for each junction (filled circles). Four junctions in which the entire junctional area was saturated are indicated with unfilled circles (these junctions were not included in the analysis of the mean). The mean (vertical line) and standard deviation (horizontal line) are indicated for each group. Of junctions in which 30% or less of the junctional area was focally blocked, 15 of 20 underwent synapse elimination. In contrast, of junctions in which 30% or more of the junctional area was blocked, only 4 of 30 showed evidence of loss. None of the junctions in which greater than 41% of the junctional area was blocked underwent synapse loss. *b*, The percentage of junctions which underwent synapse elimination (circles) is plotted against the per cent of junctional area focally blocked. As the percentage of blocked junctional area increases, the probability of loss decreases. These data indicate that inactive synaptic regions are at risk for elimination only when some threshold level of activation remains in the rest of the junction.

sist (see for example Fig. 2). The results thus suggest that the ensemble activity of a postsynaptic region is the critical factor that determines its fate. Activity-dependent accumulation of a chemical signal (such as calcium) or production of an electrical membrane signal (such as voltage) may, if it reaches some threshold, protect any receptors within an active region whether they themselves are active or not. This may explain why despite the gradient of blockade produced by α btx application there was ultimately a sharp boundary between the lost and maintained regions.

In addition to AChR loss, focal postsynaptic blockade also causes the permanent withdrawal of the overlying nerve terminal. Focal blockade could cause synapse loss by restricting trophic feedback from the postsynaptic cell so that some terminals are nourished as adjacent terminals are deprived. Alternatively, it may only be necessary that the adhesive bonds that affix a nerve terminal to the basal lamina overlying postsynaptic sites are eliminated within the focally blocked area. A role for adhesion during periods of muscle growth²⁰ and atrophy²¹ suggests that the adhesive bonds between pre- and postsynaptic elements may be critical to nerve terminal maintenance. Proteases have been implicated in the elimination process^{48,49} and could potentially break these adhesive bonds. The ability to predict exactly where synapse elimination is going to occur using the focal blockade approach should allow a careful analysis of these possibilities. □

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Campomelic dysplasia and autosomal sex reversal caused by mutations in an *SRY*-related gene

Jamie W. Foster, Marina A. Dominguez-Steglich^{*†}, Silvana Guioli, Cheni Kwok, Polly A. Weller, Milena Stevanović, Jean Weissenbach[‡], Sahar Mansour^{*§}, Ian D. Young^{*}, Peter N. Goodfellow^{||}, J. David Brook^{*†} & Alan J. Schafer

Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2 3EH, UK

^{*} Centre for Medical Genetics, City Hospital, and [†] Department of Genetics, Queens Medical Centre, University of Nottingham, Nottingham NG7 2UH, UK

[‡] Laboratoire Génétique, 1 rue de l'Internationale, Evry 91002, France

[§] Institute of Child Health, Guilford Street, London WC1N 1EH, UK

Induction of testis development in mammals requires the presence of the Y-chromosome gene *SRY*. This gene must exert its effect by interacting with other genes in the sex-determination pathway. Cloning of a translocation chromosome breakpoint from a sex-reversed patient with campomelic dysplasia, followed by mutation analysis of an adjacent gene, indicates that *SOX9*, an *SRY*-related gene, is involved in both bone formation and control of testis development.

THE *SRY* (sex-determining region Y) gene is a dominant inducer of testis development in mammals^{1,3}. The *SRY* protein contains a DNA-binding motif, termed an HMG box, which is present in several transcription factors. The *SRY* HMG box binds to specific DNA sequences, suggesting that it affects transcription of genes 'downstream' in the testis development pathway. Since the discovery of *SRY*, many other genes have been identified that encode proteins with related HMG boxes³. Those that encode proteins with more than 60% similarity to the *SRY* HMG box region have been termed *SOX* genes.

The identification and cloning of *SRY* depended on the investigation of the genomes of patients with sex reversal syndromes, some with chromosomal rearrangements. In addition to *SRY*, several autosomal and one X-linked loci have also been linked with the failure to develop a testis⁴⁻⁹. Duplications of the X chromosome short arm cause XY female development⁴. The sex reversal in these patients results from the presence of two active copies of *DSS* (dosage-sensitive sex-reversal gene) which maps to a 160-kilobase (kb) region of Xp21 (ref. 8). Autosomal loci on chromosome 9p and on 10q have been implicated by chromosomal deletions in XY females^{6,7}. It is not known if the sex reversal in these instances is due to monosomy for dosage-sensitive genes or whether the deletions reveal recessive mutations. A third autosomal locus, *SRA1*, is on chromosome 17 (ref. 10) and, in this case, the sex reversal is associated with campomelic dysplasia (CD), a skeletal malformation syndrome. The most obvious feature of CD is congenital bowing and angulation of the long bones which is often found combined with other skeletal abnormalities and defects of cartilage formation¹¹. Patients usually die in the first months of life from respiratory failure, but the severity of the phenotype is variable and a few patients survive into adult life. So far there have been at least 121 reported cases of CD. Of those that have been karyotyped, 24 are 46,XX females, 14 are 46,XY males, 36 are 46,XY females, with a gradation of genital defects^{10,12,13}. The remaining 47 non-karyotyped cases show a skewed sex ratio of 31:16 in favour of females. Some of the sex-reversed cases examined histologically

exhibit gonadal dysgenesis, implying that the gene(s) responsible for CD also plays a part in testis formation.

The inheritance pattern of CD is not obvious. Many reviewers have concluded that autosomal recessive inheritance is likely¹⁴, although it is difficult to distinguish this pattern from autosomal dominant inheritance with variable penetrance. Similarly, it is not clear whether the bone malformation and sex reversal are caused by mutation of a single gene or of a pair of linked genes in a contiguous gene syndrome. Five chromosomal rearrangements associated with CD and sex reversal have been reported that localize the gene(s) responsible to the long arm of human chromosome 17 (refs 10, 12, 15). This localization has been refined to 17q24.1–q25.1 with *GH* and *TK* as flanking markers¹⁰. We have constructed a high-resolution map across this 20-megabase (Mb) region using a panel of whole-genome radiation hybrids. The map has been used to position the translocation breakpoint from a 46,XY,t(2;17)(q35;q23–24) sex-reversed CD individual (patient E.)¹². We have found an *SRY*-related gene, *SOX9*, adjacent to the translocation breakpoint. Mutation analysis and DNA sequencing have been used to demonstrate that both CD and sex reversal can be caused by mutations in *SOX9*.

High-resolution map of 17q24.1–q25.1

Radiation hybrid mapping allows the integration of different types of markers into a single map^{16,17}. We used the polymerase chain reaction (PCR) to screen DNA samples from a panel of 129 whole-genome-radiation fusion hybrids, with a total of 38 markers across the region from *GH* to *TK* on chromosome 17. The same markers were then tested on the somatic cell hybrid B1, which was constructed by fusing mouse L cells with fibroblasts from patient E., a sex-reversed CD patient. The hybrid B1 retains the human translocation chromosome 2pter–q35:17q23–qter in the absence of the normal human chromosome 17 and of the reciprocal translocation chromosome. Chromosome 17 markers present in B1 must be located distal to the breakpoint (that is, between the breakpoint and the end of the long arm of chromosome 17), whereas markers missing from the hybrid must be located proximal to the breakpoint. From this analysis, we deduced that the microsatellite marker *D17S970* was the

|| To whom correspondence should be addressed.

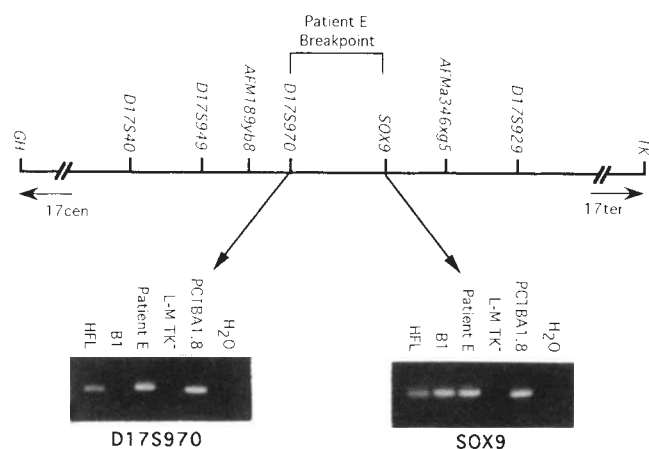


FIG. 1 Radiation hybrid map of 17q across the translocation breakpoint in patient E. STS markers are written vertically above a solid bar representing genomic DNA. The markers flanking the translocation breakpoint are indicated. Below, flanking STS markers *D17S970* and *SOX9* tested on the B1 hybrid by PCR showing their absence/presence respectively. B1 is an L-M TK⁻ somatic cell hybrid containing the translocation chromosome 2pter-q35:17q23-qter from patient E. PCTBA1.8 is a mouse somatic cell hybrid containing human chromosome 17 only; HFL is a human fibroblast; L-M TK⁻ is a mouse fibroblast.

METHODS. The whole-genome-irradiation and fusion hybrids (WG-RH) were constructed by fusing A23 hamster fibroblasts with irradiated (6,000 rads) HFL human fibroblasts¹⁷. The STS marker was determined using the RHMAP programmes⁴². PCR reactions were performed with 50 ng genomic DNA, 1.5 mM MgCl₂ (2.5 mM MgCl₂ for *SOX9* primers), 50 mM KCl, 0.1% Triton-X100, 10 mM Tris-Cl (pH 8.5), 1.5 U *Taq* polymerase and 1 µM each primer. Thermocycling parameters were 94 °C for 60 s, followed by 35 cycles of 94 °C for 30 s; 55 °C for 30 s; 72 °C for 60 s, then 5 min at 72 °C. The presence or absence of each STS in each WG-RH was determined by electrophoresis through ethidium bromide stained agarose gels. Primer sequences: AFMa346x5-A, 5'-CCAAAGTCCTAAAGGTGGG-3'; AFMa346x5-B, 5'-TTTCAGGCAATAAGGAG-3'; AFM189y8-A, 5'-TGGCAATCTAACAGATGAGA-3'; AFM189y8-B, 5'-TCNCAAATGTCATATATCCA-3'; *SOX9*-A, 5'-AGTCCAGATTGACTGGAACACA-3'; *SOX9*-B, 5'-GCAATAAGATACTAATATGTAGAG-3' *D17S40*-A, 5'-GTCAGCAGAAATCCTAAAGG-3'; *D17S40*-B, 5'-GACTAATGCCGATGGTTAAG-3'. The other primer sequences are available through the Genome Data Base.

closest proximal marker to the breakpoint and the gene *SOX9* was the flanking distal marker (Fig. 1). Assuming a distance of ~20 Mb between *GH* and *TK*, the radiation hybrid map can be used to estimate the distance between *D17S970* and *SOX9* as 1.2 Mb.

Translocation breakpoint localized

The markers flanking the translocation breakpoint were used to screen the ICRF¹⁸ and CEPH YAC libraries¹⁹ and a YAC contig was constructed based on STS content (Fig. 2). Probes from the ends of the YACs were isolated and tested back on hybrid B1 DNA as well as the other YACs to verify the contig. The YAC ICRFy900D0292, which was identified by the *SOX9* probe, yielded an end clone, D0292-R, that failed to hybridize with hybrid B1 DNA. This result placed the translocation breakpoint in the region between *SOX9* and D0292-R. Analysis of ICRFy900D0292 by pulsed-field gel electrophoresis showed that these markers were separated by 105–120 kb (data not shown).

A cosmid contig of the region between *SOX9* and D0292-R was constructed by screening the ICRF chromosome 17 cosmid library¹⁸ with inter-Alu PCR products derived from one of the YACs (946 E12) that spans the region. Inter-Alu positive cosmids were tested with markers flanking the translocation breakpoint, and these served as starting points for a cosmid walk. A contig was assembled using isolated cosmid ends to identify overlapping cosmids from the YAC Alu-PCR positive

cosmid set (Fig. 2). The end clones were mapped back onto the hybrid B1, and one of these detected the breakpoint in patient E. and hybrid B1 on Southern blots of *Bam*HI-digested DNA (data not shown). The distance from the breakpoint to the *SOX9* open reading frame is 88 kb.

Characterization of the *SOX9* gene

Transcripts corresponding to the human *SOX9* gene were isolated previously by screening a testis complementary DNA library at high stringency with a *SOX4* HMG-box probe²⁰. The isolated cDNAs were identified as *SOX9* based on similarity to the published partial sequence containing the mouse *Sox-9* HMG box region²¹. We have assembled a composite transcript of 3,934 base pairs (bp) using sequence obtained from cDNA clones isolated from three independent libraries (Fig. 3a). Comparison of this sequence with the corresponding genomic DNA revealed the presence of two introns (Fig. 3a, b), the boundaries of which have canonical splice site junctions. *SOX9* is the first *SOX* gene reported to contain introns; other *SOX* genes studied at the genomic level are single exon genes^{1,20,22,23}. The 3' region of the composite cDNA sequence contains a potential polyadenylation signal located 19 bp upstream from a terminal polyadenosine tract. The cDNA sequence diverges from the genomic sequence at the poly(A) tract, indicating that the cloned cDNA contains the 3' end of the *SOX9* transcript. The composite cDNA contains an open reading frame (ORF) with an HMG box and four potential start codons. Using the most 5' methionine as the translation start site, a polypeptide of 509 amino acids is predicted (Fig. 3a). This methionine is located 125 bp downstream of an in-frame stop codon, strongly suggesting that the complete ORF is contained within the cloned cDNA sequences. Northern blot analysis using a *SOX9* cDNA probe detects a transcript of ~4.5 kb in total cytoplasmic RNA from adult testis, adult heart and fetal brain (data not shown). The *SOX9* protein HMG box domain at amino acids 104–182 shares 71% similarity with the SRY HMG box and the C-terminal third of the protein has a proline- and glutamine-rich region, similar to activation domains present in some transcription factors²⁴. DNA and protein sequence database searches and subsequent sequence alignment with the *SOX9* HMG box identified mouse *Sox-9*, *Sox-8* and *Sox-10* as the most related sequences at 100, 98 and 93% predicted amino-acid identity, respectively. The same searches using sequences located outside the HMG box did not detect any significant matches in the databases.

Initial localization of *SOX9* using a monochromosomal somatic cell hybrid mapping panel, followed by sublocalization using chromosome-17 deletion hybrids mapped the gene to 17q23-qter (Fig. 3 legend). This localization was refined to 17q24 by fluorescence *in situ* hybridization.

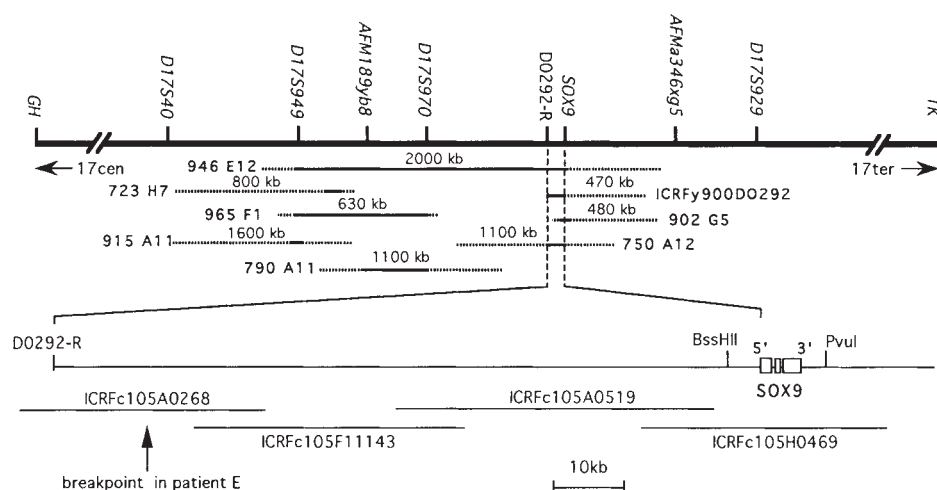
Mutation analysis of *SOX9*

The juxtaposition of *SOX9* and the translocation breakpoint in patient E. prompted us to test for mutations in this gene in DNA samples from patients with clinically confirmed CD. Initial screening was performed using a single-strand conformation polymorphism (SSCP) assay. Primers were designed to amplify the known coding sequences and intron/exon junctions in overlapping fragments of ~150 bp. Fragments that gave altered SSCP patterns (unique SSCP conformers) were cloned into plasmid vectors and sequenced. Nine patient samples were investigated; these samples yielded six heterozygous mutations. We now describe three patients in detail.

Patient S.H. (46,XX female) (ECACC no. DD1813). This patient was delivered at term with typical features of CD: micrognathia, hypoplastic scapulae, bilateral talipes equinovarus, hypoplastic cervical vertebrae, bowing of the long bones, and eleven pairs of ribs. Cloning and sequencing of a unique *SOX9* SSCP conformer for this individual revealed a cytosine-to-thymidine base transition (nucleotide 583) which introduces a stop codon at amino-acid position 195 of the predicted 509-

FIG. 2 Relationship between the chromosome-17 radiation hybrid map, YAC contig and cosmid contig for the region of the Patient E. translocation breakpoint. Markers are indicated vertically above a solid bar representing genomic DNA. YACs are positioned below: solid bars indicate confirmed marker content, dashed lines represent the possible extent of the YAC. Sizes indicated are for the entire YAC and may include non-chromosome 17 sequences present as a result of chimaerism. The STS markers *D17S970* and *D17S949* had already been used to screen the CEPH library as part of the Genethon and Whitehead/MIT Genome Center mapping projects. The cosmid walk is shown below an expansion of the breakpoint region genomic DNA. The organization and orientation of *SOX9* are indicated. ICRF Reference Library YAC and cosmids¹⁸ are indicated as such, all other YACs are from Centre d'Etude du Polymorphisme Humain¹⁹.

METHODS. YAC and cosmid ends were isolated by vectorette PCR⁴³



using the published YAC primers and cosmid vector (Lawrist4) primers LAW4L: CGCCTCGAGGTGGCTTATCG and LAW4R: ATCATACACATACGATTAGGTGAC.

amino-acid sequence (Fig. 4). Both parents of this patient were screened by SSCP for this portion of *SOX9* and neither showed an aberrant shift (Fig. 4). In addition, DNA samples from over 100 unaffected individuals were screened by SSCP for this region of *SOX9*. No anomalous shifts were seen in any normal individual. This is a *de novo* mutation.

Patient A.H. (46,XY female) (NIGMS no. GM01737). This sex-reversed individual was delivered at term with a full spectrum of CD symptoms, including short bowed limbs, small scapulae and characteristic facial features²⁵. Normal external female genitalia were present and the gonads were poorly differentiated with a substantial number of germ cells. Cloning and sequencing of the unique SSCP conformer for this patient (Fig. 4) identified a single G insertion in a series of six Gs (nucleotides 783–788) contained within codons 261–263 of *SOX9*. The resulting frameshift introduces a premature stop codon such that a 294-amino-acid truncated protein would be translated. Parental DNA of this patient could not be obtained. To investigate the possibility that this mutation occurs in unaffected individuals, SSCP was performed on this region of *SOX9* in more than 100 individuals without CD. No shifts corresponding to the patient A.H. unique conformer were found.

Patient G. (46,XY female). Following ultrasound findings of short limbs and cystic hygroma, this fetus was aborted at 17 weeks. Clinical and radiological features include micrognathia, bowing of the limbs, hypoplastic scapulae, dislocated hips and eleven pairs of ribs. Normal female genitalia were present and the ovaries appear histologically normal with oocytes. The mutation found in the unique SSCP conformer from this patient is the result of a 4-bp insertion following amino-acid 286 (nucleotide 858) of the predicted protein sequence (Fig. 4). This frameshift introduces a premature stop at the same position as in patient A.H. SSCP analysis of this region of *SOX9* from both parents revealed a normal *SOX9* shift (Fig. 4). This is a *de novo* mutation.

Mutations identified in the other three CD patients include a transition in the splice acceptor consensus dinucleotide in an XY female patient and transitions causing two different amino-acid substitutions in an XY male and an XX female.

Discussion

We have used a positional cloning strategy to define a breakpoint from a patient with both CD and autosomal XY sex reversal. The open reading frame of *SOX9*, an *SRY*-related gene, is located 88 kb distal to the breakpoint on chromosome 17. We have found mutations in single alleles of *SOX9* in six of nine campo-

melic dysplasia patients examined. The three mutations described in detail here would be expected to destroy gene function: two mutations cause frameshifts that lead to premature chain termination and loss of one third of the protein, and one mutation causes a premature termination that truncates the protein at 40% of its predicted length. SSCP analysis of both parents of two of the patients revealed the absence of the mutation present in their offspring. The *de novo* appearance of a mutation in a sex-reversed CD patient establishes that alterations in *SOX9* can cause both CD and autosomal sex reversal.

The precise relationship between the translocation breakpoint and *SOX9* is at present unclear. The *SOX9* transcript in adult testis, adult heart and fetal brain is ~4.5 kb, but the isolated cDNAs correspond to 3.9 kb of the transcript, leaving ~600 bp of untranslated sequence unaccounted for. The genomic arrangement of *SOX9* is such that the 5' end is oriented towards the chromosome-17 centromere and closest to the breakpoint. It is possible that one or more exons are present 5' to the known exons, and that these are disrupted by the translocation. Alternatively, the translocation may disrupt expression by a more subtle mechanism^{26–28}. It is striking that several of the CD translocation patients have survived early childhood and the disease may be milder in these individuals²⁹.

Campomelic dysplasia has been described as an autosomal recessive or even X-linked disease, although a few cases are more consistent with a dominant disorder^{30–32}. Our results support the suggestion that CD is an autosomal dominant disease. We have not detected mutations in both *SOX9* alleles of any patient, in spite of having performed SSCP across >70% of the open reading frame. The predicted loss of gene function in these mutants, together with the absence of mutations in both alleles implies that the dominance is due to haplo-insufficiency rather than gain of function. Dosage sensitivity is often a feature of regulatory genes and has been described for several sex determination systems, including the mammalian pathway^{8,33}.

A prediction for autosomal dominance of *SOX9* mutations is that deletions resulting in monosomy of 17q should cause CD. Such deletions are very rare, presumably because of an associated lethality, and have nearly always been reported associated with a ring chromosome. Interestingly, in a single reported 17q deletion not associated with a ring chromosome, the patient exhibited a number of physical features that occur in CD, including angulation of the lower limbs³⁴. Cases diagnosed as CD have a wide range and severity of associated phenotypes, including 'acampomelic' CD and the suggestion of long-bone and short-

FIG. 3 a, Nucleotide and predicted amino-acid sequence of SOX9. Numbering is with respect to the A in the first methionine codon of the open reading frame. An in-frame 5' stop codon and the predicted termination stop codon are in bold. The HMG box is boxed and the proline- and glutamine-rich region is underlined. The locations of the introns are indicated with arrows and a potential polyadenylation signal is indicated by bold italic letters. b, Genomic organization of the SOX9 gene. The solid bar represents genomic DNA. The SOX9 exons are boxed and the HMG box cross-hatched. E, *EcoRI*. Below, the cDNA is represented as an open box with the HMG box cross-hatched. The positions of the introns are indicated.

METHODS. Initial cDNA clones were obtained by screening a λ gt11 human testis library (Clontech) using a SOXA box probe²⁰. A composite transcript was determined from these overlapping clones and from further clones obtained from an HT1080 (fibrosarcoma) cDNA library (gift from D. L. Simmons) and a human fetal brain library (HGMP Resource Centre, Harrow). Sequencing was performed using the dideoxy chain termination method. The location of the intron/exon boundaries was determined by restriction mapping of genomic and cDNA clones and by comparison of the genomic and cDNA sequences. Initial localization of the SOX9 cDNA to chromosome 17 was determined by probing a somatic cell hybrid panel. Sublocalization to 17q23-qter was determined using a panel of chromosome-17 deletion hybrids, including PCTBA1.8, TRID62, PLT8, PJT2A1 and DCR1 (ref. 44), and refined to 17q24 by fluorescence *in situ* hybridization to normal human metaphase spreads.

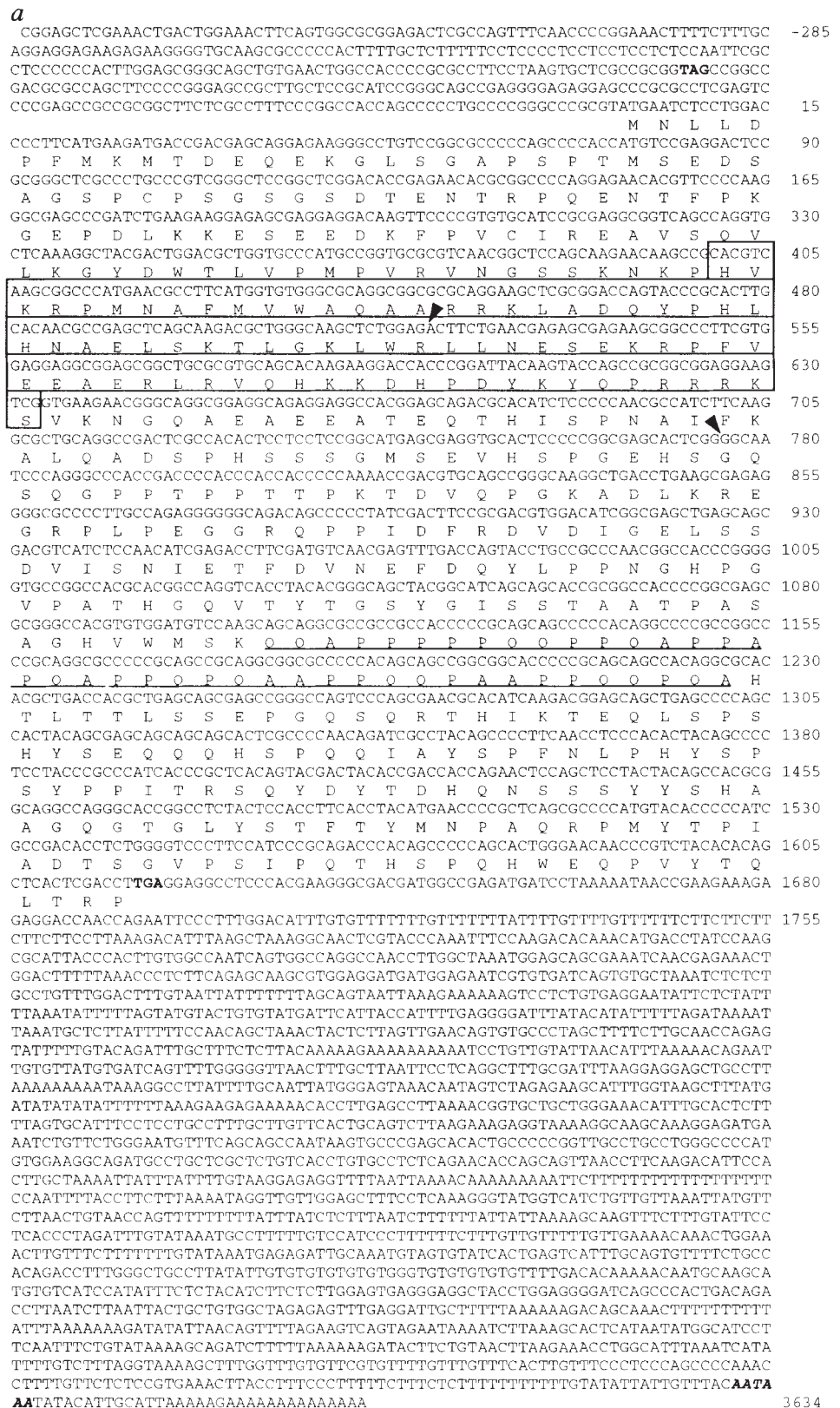
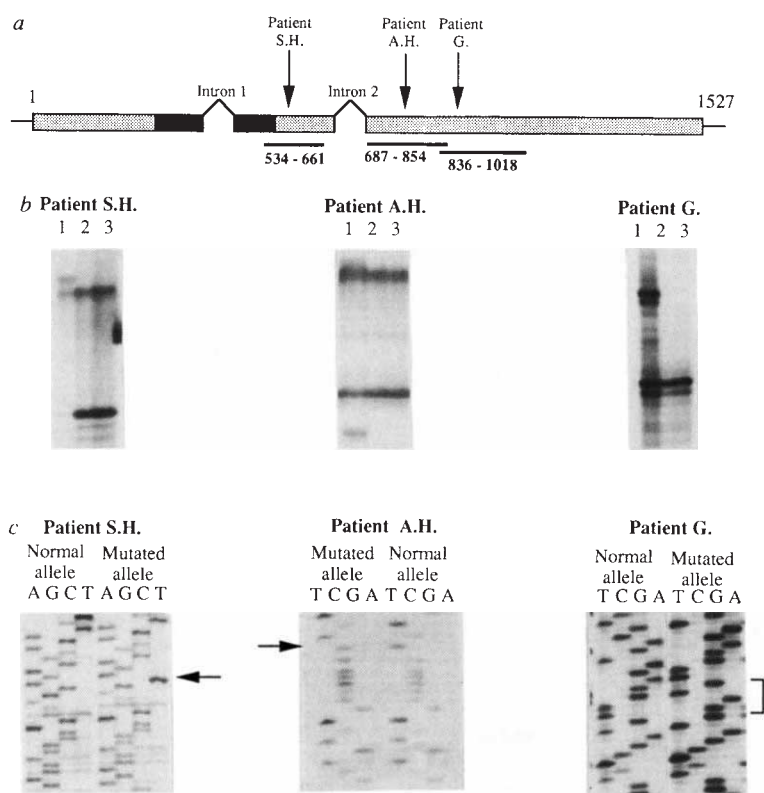


FIG. 4 Single-strand conformation polymorphism (SSCP) and sequence analysis of *SOX9* in campomelic dysplasia patients. **a**, *SOX9* open reading frame (shaded boxes) showing the HMG box (heavy shading). Numbers indicate nucleotide sequence beginning with the A of the first methionine, with introns occurring after nucleotides 431 and 685. Solid bars below indicate regions of the ORF generating unique SSCP conformers. Positions of mutations are indicated by arrows. **b**, SSCP using primers indicated in **a**. Lane 1, patient DNA. For patients S.H. and G., lanes 2 and 3 are DNAs from father and mother, respectively. For patient A.H., lanes 2 and 3 are DNAs from unrelated (normal) individuals. **c**, Sequencing gels of normal and mutated patient alleles. The position of each mutation is indicated. Sequence for patients S.H. and A.H. is that of the coding strand; for patient G., the sequence is of the non-coding strand.

METHODS. Primer sequences: 534, 5'-GAGGAAGTCGGT-GAAGAAC-3'; 661, 5'-TCGCTCATGCCGAGGAGGAG-3'; 687, 5'-GCAATCCAGGCCACCGAC-3'; 854, 5'-TTGG-AGATGACGCTGCTGCTC-3'; 836, 5'-GCAGCGACGTCATCT-CCAAC-3'; 1018, 5'-GCTGCTTGGACATCCACGT-3'. PCRs (10 μ l) were performed as for Fig. 1, with the non-radioactive dCTP concentration reduced to 1/10 and the addition of 0.05 μ l [α -³²P]dCTP (1,000–3,000 Ci mmol⁻¹, 10 mCi ml⁻¹) and 0.2 μ M of each primer. Reactions were cycled for 30 s at 94 °C, 30 s at 65 °C (534–661 and 836–1,018) or 70 °C (687–854), 45 s at 72 °C for 35 cycles. PCR products were denatured by adding 10 μ l 0.2% SDS, 20 mM EDTA then 10 μ l 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol and heating to 100 °C for 5 min. 2 μ l were loaded onto 6% acrylamide:bisacrylamide (37.5:1), 5% glycerol gels. Electrophoresis was carried out at 25 W at 4 °C. PCR products from duplicate reactions were subcloned and at least 10 clones from each were sequenced by either the dideoxy chain termination method or by DyeDeoxy Terminator Cycle Sequencing (ABI). DNA



profiling of each family using 12 chromosome-8 microsatellite markers (heterozygosity >70%) showed no discordant results between parents and offspring.

bone varieties¹¹. It will be of interest to determine the extent of *SOX9* involvement in all cases diagnosed as CD.

By analogy with *SRY*, it has been suggested that *SOX* genes might act as transcription factors in developmental control pathways. Some *SOX* proteins show sequence-specific binding^{35,37} and the C-terminal third of the *SOX9* protein has a proline- and glutamine-rich region, similar to activation domains present in some transcription factors²⁴. This region would be missing in products translated from the mutated sequences present in the patients described here.

SOX9 is not the first mammalian gene to be shown to have a dosage-sensitive role in sex determination. *DSS* causes male-to-female sex reversal, with varying degrees of masculinization, when present in two copies in 46,XY individuals. Absence of *DSS* is compatible with male development in the presence of *SRY*, but it is not known if it is compatible with female development in 46,XX individuals. Because of the importance of *SOX9* in bone formation, it is likely that nullisomy for *SOX9* is lethal. *SOX9* monosomy is compatible with ovarian development³⁴ and trisomy for 17q, including the region containing *SOX9*, has not been associated with sex reversal³⁸. The cause of the variability of sex reversal associated with CD remains to be determined. There is no obvious correlation between the severity of the skeletal anomalies and the incidence of sex reversal²⁹. The presence or absence of sex reversal in XY individuals may be determined by the nature of the mutation, or could lie in allelic differences at other loci.

The dosage sensitivity of *SOX9* in sex determination and its sequence similarity to *SRY* suggest a possible evolutionary relationship between the two genes. It is plausible that a primordial dosage-dependent sex-determination system evolved into a dominant induction system by alteration of *SOX9* or another *SOX* gene³⁹. The mutated gene could function as a dominant inducer by becoming constitutively expressed and thus, when

present, increasing dosage to be above a threshold required for male development.

There is a large body of indirect evidence suggesting that the sex-determining function of *SRY* is expressed in pre-Sertoli cells in the developing gonadal ridge³. *SOX9* could be required in these cells, and *SRY* and *SOX9* interactions may be required for full cell function. Another possibility is that *SOX9* expression is required in a cell type that interacts with *SRY*-expressing pre-Sertoli cells to form testis. It is known that mesenchymal cells migrate from the mesonephros underlying the genital ridge and that these migratory cells are required for testis formation⁴⁰. Mesenchymal cells give rise to both bone and cartilage forming cells⁴¹ and this might provide the link between CD and sex reversal. The identification of *SOX9* as a gene mutated in both CD and autosomal sex reversal provides new tools for studying bone formation and sex determination. □

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LETTERS TO NATURE

A high-resolution image of atomic hydrogen in the M81 group of galaxies

M. S. Yun^{*†}, P. T. P. Ho^{*} & K. Y. Lo[‡]

^{*} Harvard-Smithsonian Center for Astrophysics, 60 Garden Street, Cambridge, Massachusetts 02138, USA

[†] California Institute of Technology, Robinson 105-24, Pasadena, California 91125, USA

[‡] Astronomy Department, University of Illinois, Urbana, Illinois 61801, USA

It has long been recognized that interactions between galaxies are important in determining their evolution. The distribution of gas—out of which new stars are formed—is strongly affected; in particular, gas may be concentrated near the nucleus, leading to a burst of star formation^{1–4}. Here we present a map of atomic hydrogen (H I) in the nearest interacting group of galaxies (that dominated by M81), obtained by combining 12 separate fields observed with the Very Large Array. The H I that surrounds M81, M82 and NGC3077 (the most prominent galaxies in the group) is dominated by filamentary structures, clearly demonstrating the violent disruption of this system by tidal interactions. These observations should have detected all H I complexes more massive than 10^6 solar masses, meaning that our map contains all structures that might evolve into new dwarf galaxies.

With the Very Large Array (VLA) in the most compact D configuration, four fields centred around M82 were observed (3 h per field) on 24 and 25 January 1990, and eight additional fields including M81 and NGC3077 were observed (2 h per field) on 29 April and 1 May 1991, using all 27 telescopes. The achieved angular resolution of ~ 1 arcmin corresponds to a resolution of ~ 1 kpc at the systemic distance of 3.6 Mpc (ref. 5). Quasars 3C48 and 3C286 were used to set the flux-density scale and to calibrate the instrumental response across the bandpass of 5.125 MHz. The VLA calibrator 0831+557 was also observed to calibrate short-term variations of the system performance. With a spectral resolution of 48.8 kHz (10.3 km s^{-1}), we covered the full velocity range ($\sim 600 \text{ km s}^{-1}$) of the entire map by shifting the centre of our spectral window accordingly.

Figure 1 shows the H I distribution in the M81 group; this H I image of the 150 kpc region is at 1-kpc spatial resolution. The comparison of the H I and the optical images shows that although this system is a favourite testing ground for the density-wave theory and disk dynamics⁶, active Seyfert nuclei^{7,8}, as well

TABLE 1 Observed properties for the main H I features of the M81 group

Feature	$N_{\text{H I}}$ (10^{21} cm^{-2})	$M_{\text{H I}}$ ($10^9 M_{\odot}$)	$M_{\text{H I}}(\text{ADS})$ ($10^9 M_{\odot}$)	ΔV (km s^{-1})
M81	10.6 ± 0.2	2.63 ± 0.52	2.19 ± 0.22	30–45
M82	10.3 ± 0.2	0.75 ± 0.15	0.72 ± 0.07	40–140
NGC3077	10.7 ± 0.2	0.65 ± 0.13	1.00 ± 0.10	30–40
Concentration I	7.8 ± 0.2	0.29 ± 0.06	$0.20 \pm 0.05^*$	45–100
Concentration II	5.1 ± 0.2	0.24 ± 0.05	—	25–30†
South tidal bridge	2.4 ± 0.2	0.24 ± 0.05	—	10–30
North tidal bridge	1.6 ± 0.2	0.19 ± 0.04	—	20–30

Symbols used: $N_{\text{H I}}$, H I column density; $M_{\text{H I}}$, total H I mass; $M_{\text{H I}}(\text{ADS})$, total H I mass within the Holmberg radius measured with a 76-m telescope (angular resolution $\theta \approx 4$ arcmin) by Appleton, Davies and Stephenson¹⁵; ΔV , linewidth, full-width at half-maximum.

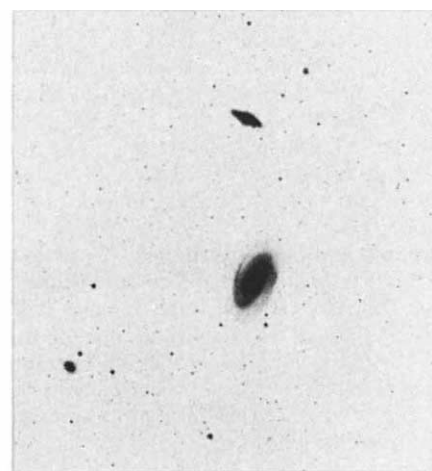
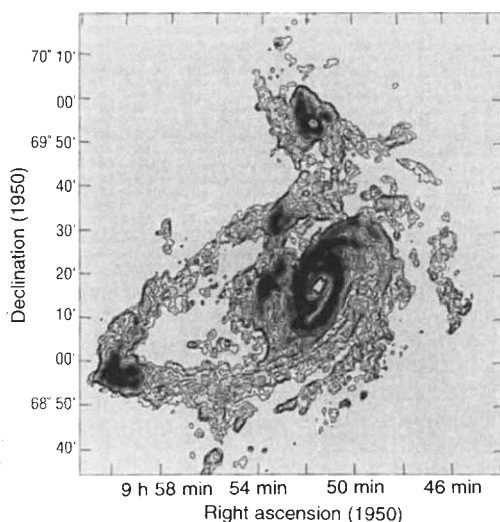
* H I mass estimated for Ho IX by Appleton, Davies and Stephenson¹⁵.

† Two separate velocity components seen, each with $\Delta V \approx 25$ –30 km s^{-1} (80–140 km s^{-1} , full-width at zero intensity).

as starburst activities⁹, little sign of tidal interactions can be seen in the optical image. Previous H I studies have suggested tidal interactions^{10–17}. The remarkable result here is the extensive array of filamentary tidal features threading all three galaxies, dominating the H I distribution. Each galaxy has a concentration of gas surrounding it, with the ‘south tidal bridge’ connecting M81 and NGC3077, and the ‘north tidal bridge’ connecting NGC3077 and M82. This is the first time that the north tidal bridge is traced in its full extent. The physical properties of the main H I features are summarized in Table 1. We will now discuss our observations of the various features found in the H I and optical maps.

M81. In the optical image, M81 appears to be a two-arm spiral. In the inner disk, the H I spiral arms trace the optical arms very closely. Moving beyond the optical disk, more H I arms can be seen which appear to be less tightly wound. West of the nucleus, one of the H I arms appears to wrap around to the east connecting with concentration I. East of the nucleus, two H I arms seem to continue on as two long spirals over 180° in angular extent, with the inner one continuing onto the south tidal bridge. The H I kinematics are shown in Fig. 2. Within the central 20 arcmin (20 kpc) around M81, the kinematics (the regular spider-like pattern for the isovelocity contours) show circular rotation. That the contours become progressively further apart away from the nucleus, shows differential rather than rigid-body rotation, that is, rotation is slower further out. This drop-off in circular rotation velocity with radius, also known as a rotation curve, was studied by Kent¹⁸. He found that the mass of the large bulge and the disk alone is sufficient to reproduce the observed rotation out to a radius R of 20 kpc (the only system in his sample of 16

FIG. 1 Right, Palomar sky survey image (in the red band) of M81 triplet. Going from north (top) to south (bottom), the three galaxies are M82, M81 and NGC3077. Other than the optical filaments seen along the minor axis of M82, there is little hint of unusual activities in these galaxies in stellar light. Left, Integrated H I map of M81 group from the new D-array VLA mosaic observations (angular resolution $\theta = 62 \times 56$ arcsec). The mosaic of the data from 12 individual fields is made by noise-weighted linear averaging of the deconvolved (CLEANed) channel maps after removing the continuum emission and correcting for the primary beam response. Although the VLA cannot sample large structures because the interferometer resolves them, nearly all the flux from single-dish studies¹⁵ is nevertheless recovered. The data cube contains a total of 81 channels covering the velocity range of -338 to $+486$ km s⁻¹. By overlapping adjacent fields at half-beam spacings, we achieved uniform r.m.s. noise of 0.8 mJy per beam over the sampled region. Galactic H I emission is present in three channels centred at $V_{\text{LSR}} = +12$ km s⁻¹ and two channels near $V_{\text{LSR}} = -50$ km s⁻¹, where V_{LSR} is velocity with respect to the local standard of rest: for the analysis of H I emission associated with the M81 group, these channels were clipped at the 10 mJy per beam



level to remove the Galactic contribution. The linear grey-scale represents the integrated H I flux. The contours correspond to the H I column density of 1.8×10^{20} cm⁻² times 1, 2, 3, 4, 6, 10, 15, 25 and 40. A total of $5.2 \times 10^9 M_{\odot}$ of H I is detected. The H I structures of interest are: the north tidal bridge between M82 and NGC3077, the south tidal bridge between M81 and NGC3077, concentration I and II which are the dark knots 10 arcmin east and 20 arcmin northeast of M81 nucleus.

galaxies which may not require any dark matter to explain the observed rotation). Our new H I data show that the rotation velocity does not fall off fast enough, flattening near $R \approx 25$ kpc. Additional mass, perhaps in the form of a dark halo, is needed to keep the observed motions gravitationally bound. Figures 1 and 2 show that beyond 30 arcmin (30 kpc), the kinematics will be affected by interactions with M82 and NGC3077. The outer velocity patterns no longer behave as if they are extensions of the contours within the central 20 arcmin. This is most obvious for the southern and northern tidal bridges, hence justifying their names. We note that the outer Lindblad resonance for M81 is located at $R \approx 15$ kpc (refs 19–21). The outer H I arms are thus most likely tidally driven transient spiral features²². Detailed numerical simulations are needed to see if the morphology and kinematics can be produced by an interaction model.

Compared with the earlier H I studies of M81 by Gottesman and Weliachew¹⁹, and Rots and Shane²³, the H I distribution in our study is traced much further out (>30 kpc in radius) because of the multiple pointings with better sensitivity. "Concentrations I and II", the H I complexes previously identified¹⁹ as extragalactic, are now located inside the H I disk of M81. An exponential disk with a scale length of 10 kpc can model the radial surface density distribution well out to 40 kpc radius. A significant excess found at $R \geq 40$ kpc where M82 and NGC3077 are located, suggests that the H I of these galaxies dominates beyond this radius. There appears to be additional H I features located outside the mapped area, that is, the westernmost feature of the M81 disk is the tip of another H I streamer pointing southwest²⁴.

The high velocity trough and Ho IX. In the inner 25 kpc, the most significant deviation from the differential disk rotation in M81 is a ~ 20 -kpc-long strip 10 arcmin east and northeast of the nucleus. This is seen as the Ω -shaped twists in the isovelocity contours in Fig. 2, with redshifts along the minor axis of up to 200 km s⁻¹ with respect to the systemic velocity of M81 ($V = -15$ km s⁻¹). We call this the 'high velocity trough' (HVT). The associated H I mass is $3.3 \times 10^8 M_{\odot}$, mostly in concentration I, and the associated (lower-limit) energy and momentum are 7.4×10^{55} erg and $5.0 \times 10^{10} M_{\odot}$ km s⁻¹. The most likely explanation may be that these are the remnants of the recent collision

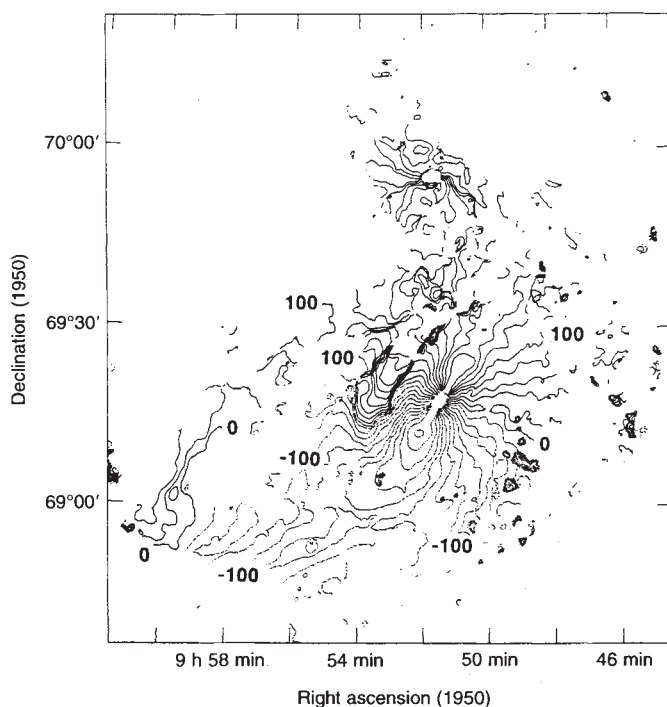


FIG. 2 Intensity-weighted mean velocity (first moment) map of H I in the M81 group. Contours are at 20 km s⁻¹ intervals, and negative velocities are marked in dotted lines. The unit of the velocity label is km s⁻¹. The isovelocity contours looking like 'spider arms' centred on M81 are the classical signatures of differential rotation. This, plus the motion of the tidally disrupted H I disk in M82, accounts for the north-south velocity gradient of this system. The differential rotation for the M81 disk appears regular for the inner 20 kpc, but the twisting isovelocity contours in the outer regions (for example, the south tidal bridge) suggest significant non-circular motions at larger radii.

between M81 and M82. The velocity of M82 matches the velocity perturbation associated with HVT both in sign and magnitude. The disk orbital period at 15-kpc radius is 5×10^8 yr, and the extent of the HVT suggests that M82 passed through the western disk of M81 near $R \approx 15$ kpc some 2×10^8 yr ago. In this scenario, the Magellanic-type dwarf Ho IX found 10 arcmin east of M81, concentration I, and the $10^6 M_\odot$ molecular complex found near the H I peak²⁵, can be explained as the resultant stellar and gaseous debris.

Arp ring and concentration II. The main body of the Arp ring²⁶, a faint halo-like optical structure found just north of M81, in fact coincides with the outer H I arm of M81. Concentration II is the brightest H I complex in the tidal bridge between M81 and M82 (Fig. 1), and it coincides with the brightest optical segment of the Arp ring. The high H I column density in this direction is due to the fact that this is the site where the tidally stripped gas from M81, the tidal streamer from M82, and the north tidal bridge intersect, each with distinct velocities and velocity widths. The Arp ring may not be a single cohesive feature.

NGC3077 and tidal bridges. The north and south tidal bridges could be interpreted as a single continuous 100-kpc-long tidal feature, which connects smoothly to one of the tidally induced spiral arms of M81. We find a local H I peak near the stellar nucleus of NGC3077 while the bulk of the $7 \times 10^8 M_\odot$ H I complex is displaced by ~ 4 kpc from the stellar peak. The H I velocity gradient across the major axis of NGC3077 is consistent with rotation. The isovelocity contours along the south tidal bridge are perpendicular to its length as expected for a tidal tail (for example, NGC7252; ref. 27), but those along the north tidal bridge, particularly near NGC3077, are along the length, as might be expected for tidally induced shearing motion, similar to that of the remnant disk around M82. This suggests that NGC3077, now appearing as a gas-poor dwarf elliptical^{28, 30}, could have been the origin for the gas complex around NGC3077 and the north tidal bridge.

M82. Earlier low-resolution studies by Cottrell¹¹ and others had interpreted the north-south overall velocity gradient perpendicular to the stellar disk as that of a tidally captured H I cloud in a polar orbit. Our recent high-resolution H I study of M82 using the VLA¹⁷ found luminous H I streamers 10–20 kpc long emerging from the warped stellar disk, and both the two-arm morphology and the smooth connection of their velocity fields onto the disk rotation are cited as the evidence for the tidal disruption of the outer H I disk of M82 by a massive companion (M81) in a polar orbit. Our study strengthens these conclusions by tracing previously discovered H I tails to greater and fainter extents (>30 kpc). The original H I disk of M82 was probably quite substantial before the tidal disruption by M81.

The synthesis of a large mosaic in H I with good angular resolution, as has been done here, can demonstrate the effect of tidal interactions on the evolution of galaxies in small groups, especially when there is little sign of close interactions in optical images. The observations presented here suggest that the presence of even a minor companion may drastically increase the apparent gaseous extent of galaxies. Further sensitive studies of other groups of galaxies will show whether the optical or H I appearances of galaxies are better indicators of the nature and history of the system. □

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Superconductivity in a layered perovskite without copper

Y. Maeno*, H. Hashimoto*, K. Yoshida*,
S. Nishizaki*, T. Fujita*, J. G. Bednorz†
& F. Lichtenberg†‡

* Department of Physics, Hiroshima University,
Higashi-Hiroshima 724, Japan

† IBM Research Division, Zürich Research Laboratory,
8803 Rüschlikon, Switzerland

FOLLOWING the discovery of superconductivity at ~ 30 K in $\text{La}_{2-x}\text{Ba}_x\text{CuO}_4$ (ref. 1), a large number of related compounds have been found that are superconducting at relatively high temperatures. The feature common to all of these materials is a layered crystal structure based on a perovskite template and containing planar networks of copper and oxygen. This raises the question of whether superconductivity can occur in layered perovskites that do not contain copper. To the best of our knowledge, no such material has been found to date, despite nearly a decade of searching. We describe here the discovery of superconductivity in Sr_2RuO_4 , a layered perovskite isostructural with $\text{La}_{2-x}\text{Ba}_x\text{CuO}_4$ (Fig. 1). Our results demonstrate that the presence of copper is not a prerequisite for the existence of superconductivity in a layered perovskite. But the low value of the superconducting transition temperature ($T_c = 0.93$ K) points towards a special role for copper in the high-temperature superconductors.

The compound Sr_2RuO_4 has been known for quite some time². It is the $n=1$ member of the homologous series expressed as $\text{Sr}_{n+1}\text{Ru}_n\text{O}_{3n+1}$, of which those members with $n=1, 2$ and ∞ have been synthesized^{2,3}. Single crystals of Sr_2RuO_4 used in the present study were grown in air by a floating-zone method⁴. They are easily cleaved, yielding plate-like crystals with (001) surfaces. Electron-probe microanalysis indicated a homogeneous distribution of the metal ions within a crystal and did not detect any impurity inclusions. All the observed peaks of powder X-ray diffraction spectra on crushed crystals are consistent with a body-centred tetragonal unit cell of the K_2NiF_4 structure with lattice parameters $a=b=0.387$ nm and $c=1.274$ nm at room temperature, in good agreement with the previous reports^{2,4}.

The low-temperature measurements were performed by using ³He refrigerator (Heliox, made by Oxford Instruments, Eynsham, UK). Figure 2 shows the alternating current (a.c.) suscep-

‡ Present address: VARTA Batterie AG, R and D Centre, 65779 Kelkheim, Germany.

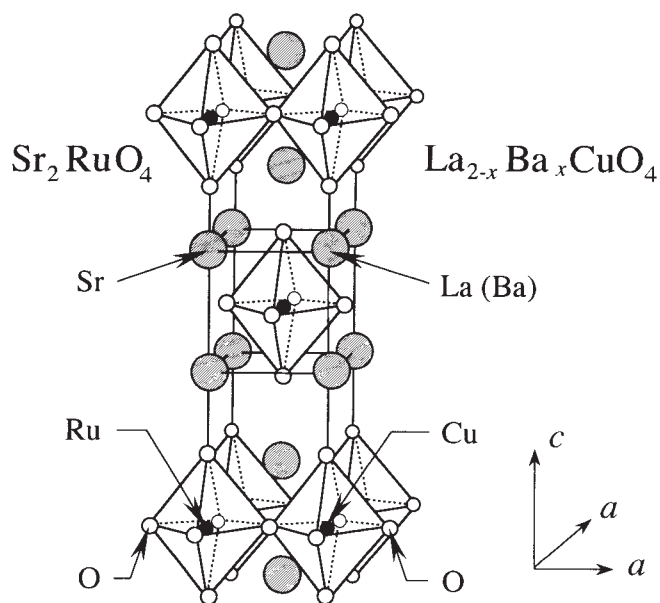


FIG. 1 Schematic crystal structure of superconductors Sr_2RuO_4 and $\text{La}_{2-x}\text{Ba}_x\text{CuO}_4$. Also shown are the directions of the tetragonal principal axes. ($b=a$ in a tetragonal structure.)

ibility of a crystal with the dimensions $3\text{ mm}^2 \times 0.2\text{ mm}$ under a magnetic field parallel to the c -axis. A strong diamagnetic signal (χ') associated with the Meissner effect was observed below 1 K. Although it did not reach a saturated value even at 0.32 K, the magnitude of χ' is already comparable to that obtained for a high-quality single crystal of $\text{La}_{2-x}\text{Sr}_x\text{CuO}_4$ ($x=0.15$) with similar volume and shape. Also shown is the dissipative component χ'' , which exhibits characteristic peaks below T_c .

Figure 3 gives the electrical resistivity below 4 K with the current both parallel (ρ_{ab}) and perpendicular (ρ_c) to the RuO_2 plane, showing the superconducting transition at T_c slightly below 1 K. The sizes of the crystals were typically $(1-2) \times 2 \times 0.1\text{ mm}^3$. Within our experimental precision of $\sim 10\text{ nV}$ for the voltage detection, zero resistivity was achieved in both ρ_{ab} and ρ_c at low temperatures. The critical-current densities were measured at 0.32 K. By increasing the measurement current,

normal-state resistivity abruptly recovered beyond $j_c=2.0$ and 0.018 A cm^{-2} , respectively within the ab -plane and along the c -axis. These values give the anisotropy ratio of 110. The resistivity curves in Fig. 3 were taken at the current densities which are half of the respective j_c s at 0.32 K. From the midpoint of the resistive transition, as well as from the diamagnetic onset, $T_c=0.93 \pm 0.03\text{ K}$ for all the as-grown crystals we have examined.

Figure 4 displays the anisotropy in the normal-state resistivity. The resistivity ratios are $\rho_c/\rho_{ab}=220$ at 290 K and 850 at 2 K. The most striking feature is the crossover in ρ_c from non-metallic to metallic behaviour with a broad maximum at $T_M \approx 130\text{ K}$, as reported previously⁴. However, improved measurements in the present study clarify subtle but important details: a change in the temperature dependence of ρ_{ab} at the corresponding temperature also becomes evident. At high temperatures, the conduction is metallic only within the plane as in $\text{La}_{2-x}\text{Sr}_x\text{CuO}_4$ with light and

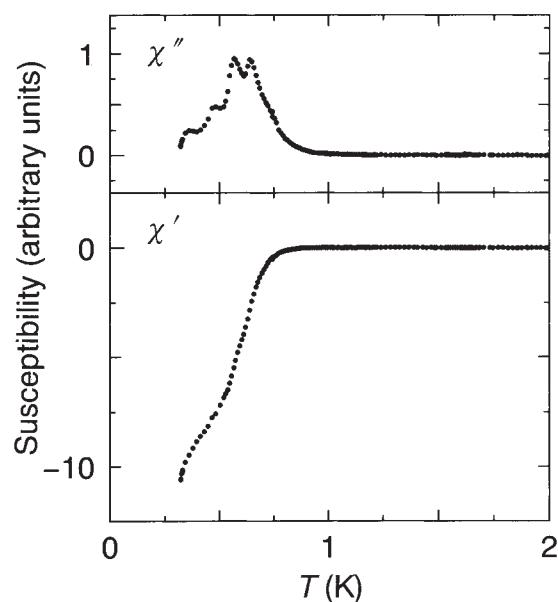


FIG. 2 The a.c. susceptibility of a single crystal of Sr_2RuO_4 measured by a mutual-inductance method at a magnetic field of $H=0.67\text{ Oe}$ (root-mean-squares value) parallel to the c -axis, and at a frequency of 1,000 Hz. Top, χ'' (imaginary part); bottom, χ' (real part).

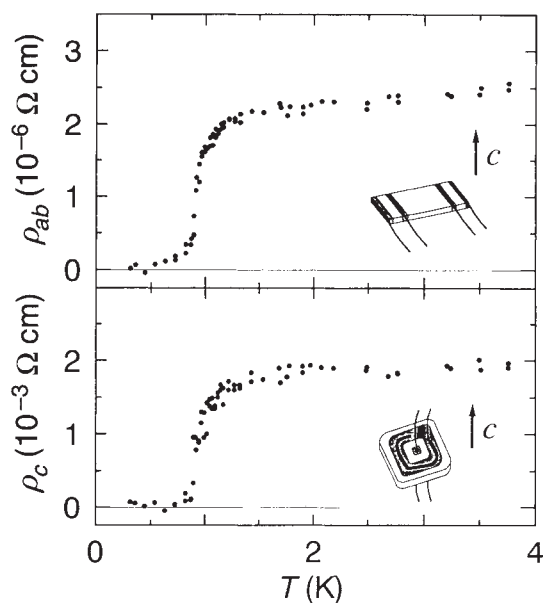


FIG. 3 Anisotropy in the resistivity ρ of Sr_2RuO_4 below 4 K. The superconducting transition is at $T_c=0.93\text{ K}$. Insets, attachment of the electrodes.

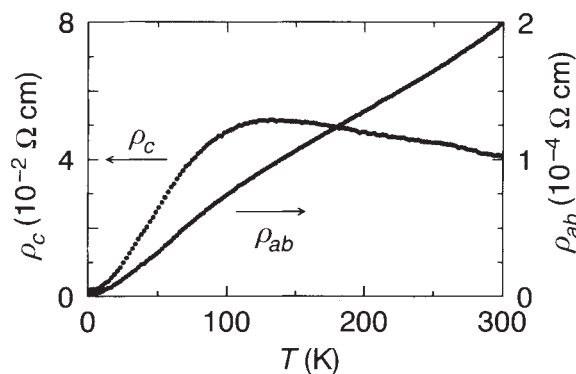


FIG. 4 Anisotropy in the resistivity of Sr_2RuO_4 below 300 K.

optimal doping; at low temperatures, the conduction becomes metallic in all three directions as in $\text{La}_{2-x}\text{Sr}_x\text{CuO}_4$ with heavy doping⁵.

We further investigated the normal-state properties mainly by using sintered polycrystalline samples. The magnetic susceptibility is characterized by an enhanced temperature-independent component, $\chi_0 = 9.7 \times 10^{-4}$ e.m.u. mol^{-1} . The Sommerfeld coefficient, or the temperature-linear coefficient in the specific heat is $\gamma = 39$ mJ K^{-2} mol^{-1} . Both of these values are enhanced several times from those of RuO_2 (refs 6, 7), which is considered as an ordinary d -band metal⁸, and are even greater than those observed in $\text{Sr}_{1-x}\text{La}_x\text{TiO}_3$ on the verge of metal/Mott-insulator transition⁹. For another comparison, $\gamma = 10$ –15 mJ K^{-2} mol^{-1} for optimally doped $\text{La}_{2-x}\text{Sr}_x\text{CuO}_4$ (M. Ido, personal communication). Moreover, the Wilson ratio¹⁰ of Sr_2RuO_4 , $R_W = (\pi^2 k_B^2 / 3\mu_B^2)(\chi_0 / \gamma) = 1.8$ (where k_B is the Boltzmann constant, μ_B is the Bohr magneton and γ is expressed in erg K^{-2} mol^{-1}), indicates that the enhancement in both χ_0 and γ are of the same origin. Although the resistivity of the polycrystalline samples were three orders of magnitude greater than ρ_{ab} , probably due to poor conductivity of the grain boundaries, it did show a strong reduction below 2.3 K and it became as small as 25% of the onset superconducting value when the sample was cooled to 50 mK in a dilution refrigerator. Nevertheless, zero resistivity was never attained in our sintered samples. The magnetic susceptibility of a sintered sample also exhibited a diamagnetic transition at ~ 1 K. The details of these results will be reported elsewhere. The origin of the discrepancies in T_c between the single crystals and the sintered samples is not understood at present.

The electronic structure of Sr_2RuO_4 has a number of similarities to that of copper oxide superconductors, although the $3d$ electrons of Cu are replaced by the $4d$ electrons of Ru. As suggested by photoelectron spectroscopy¹¹ of related compounds, the states near the Fermi level are composed of hybridized Ru- $4d$ and O- $2p$ states. The presence of strong Coulomb correlations among the electrons is reflected in their enhanced effective mass, as well as by the fact they $\text{Y}_2\text{Ru}_2\text{O}_7$ with a distorted array of corner-shared RuO_6 octahedra¹² is non-metallic because of correlation-induced electron localization¹¹.

It is well known¹³ that ρ_c of $\text{La}_{2-x}\text{Sr}_x\text{CuO}_4$ is profoundly affected by the structural transition from the high-temperature tetragonal phase to the mid-temperature orthorhombic phase. We investigated the low-temperature structure of Sr_2RuO_4 by powder X-ray diffraction of crushed crystals, but did not detect any structural transition down to 5 K or any change in the temperature dependence of the lattice parameters across T_M . This result is consistent with a previous structure refinement¹⁴ at 100 K. Therefore the origin of the sign change in $d\rho_c/dT$ with temperature in the present system appears to be purely electronic, although we still need to clarify whether a subtle (or

local) change in the structure exists. The resistivity behaviour may be explained in terms of the criterion for three-dimensional anisotropic metals as was done for copper oxide superconductors⁵, or alternatively in terms of a competition between the transfer energy along the c -axis and the thermal energy. In any case, this curious normal-state behaviour alone is worth further investigation.

There are important differences between copper oxide superconductors and Sr_2RuO_4 . First, the T_c of isostructural copper oxide superconductors is higher by a factor of 20–40 than that of Sr_2RuO_4 . This fact, however, may provide easier comparison between Sr_2RuO_4 and conventional superconductors, because the electronic contribution to various physical quantities near T_c is not overwhelmed by the phonon contribution. Second, the Cu^{2+} ($3d^9$) valence state has spin 1/2, whereas Ru^{4+} ($4d^4$) is in the low-spin state with spin 1, as demonstrated¹⁵ in $\text{Sr}_2\text{Ir}_{1-x}\text{Ru}_x\text{O}_4$. The important d - p hybridization occurs with $e_g(d_{x^2-y^2})$ orbitals in the copper oxides, in contrast to t_{2g} orbitals in Sr_2RuO_4 . Many of the existing theories for copper oxide superconductors rely upon peculiar normal states derived from the spin-1/2 state. (see, for example, ref. 16). Intriguingly, when we measured the resistivity of polycrystalline Sr_2RhO_4 , based on Rh^{4+} ($4d^5$) with spin 1/2 we found it to be metallic, with no evidence for superconductivity down to 50 mK. It therefore seems probable that superconductivity in the Ru-based compound involves a different mechanism from that operating in the copper oxides.

Last but not least, superconductivity in the copper oxides is realized only after carrier doping of an insulating parent compound such as La_2CuO_4 , whereas Sr_2RuO_4 (without any chemical doping to adjust the carrier concentration) is superconductive by itself. For the copper oxides, new states at the Fermi level are created within the correlation (charge-transfer) gap by carrier doping. In contrast, for the Ru-based oxides similar states are expected¹⁷ to evolve within the correlation (Mott–Hubbard) gap by weakening the effective strength of the electron correlation in order from non-metallic $\text{Y}_2\text{Ru}_2\text{O}_7$ with a highly distorted Ru–O network, to metallic Sr_2RuO_4 with very strong anisotropy, to metallic CaRuO_3 with itinerant-electron magnetism^{18,19} and to an ordinary d -band metal RuO_2 . This explains why carrier doping is not necessary in Sr_2RuO_4 and opens a second route to realize superconductivity in layered perovskites. Although this model needs to be substantiated further by detailed spectroscopic measurements, our search for superconductivity in Ru-based oxides has been motivated precisely by this picture. □

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An atomistic model for stepped diamond growth

Michael Frenklach*, Sergei Skokov*
& Brian Weiner*†

* Department of Materials Science and Engineering,
The Pennsylvania State University, University Park,
Pennsylvania 16802, USA

† Department of Physics, The Pennsylvania State University, DuBois,
Pennsylvania 15801, USA

THE growth of many crystalline materials occurs through lateral propagation of steps over the surface^{1–3}. A stepped texture is also characteristic of diamond grown by chemical vapour deposition (CVD)^{4–10}. Diffusion of atoms on the surface is usually held responsible for the stepped growth of metals, but it has been thought^{11–14} that the stronger bonding of adatoms should prevent this mechanism from operating in the case of diamond. Recent experiments¹⁵ have, however, indicated that surface diffusion can take place during diamond growth. We have recently shown theoretically¹⁶ that bridging methylene (CH_2) groups on the $\{100\}$ plane of diamond growing in the presence of hydrogen can migrate in a manner equivalent to surface diffusion. Here we show that this theoretical picture can be developed into an atomistic model that accounts for stepped growth of diamond. The stepped pattern can be understood in terms of the formation of surface-bound species from the gaseous precursors, followed by their migration by means of a series of surface chemical reactions involving covalent bond breaking and formation.

Our analysis is based on the evaluation of rates and equilibrium constants for elementary surface reactions. The method employed is described in our publications^{16,17}. Briefly, the reaction rate constants were calculated using non-variational transition-state theory¹⁸. The input data for these calculations were obtained from quantum-chemical calculations. The potential-energy barriers were determined in combined-force molecular dynamics calculations in which the surface is modelled by a composite cluster: the core is described by a quantum-mechanical potential, and the surrounding atoms by an empirical potential fitted to the potential of the core. The quantum region contained ~40–50 carbon atoms and the empirical region up to 600 carbon atoms. The large size of the model produces a realistic representation of surface constraints. But vibrational frequencies, the other input data required for the rate-constant

calculations, cannot be established reliably for these clusters. Hence they were determined from static quantum-chemical calculations performed on small clusters. Minimum-energy configurations in both types of calculations were obtained by the Hartree-Fock method using a PM3 semiempirical hamiltonian¹⁹, a level of theory well suited for establishing chemical reaction mechanisms²⁰.

The focus of the present study is a surface step shown schematically in Fig. 1. The step is formed by $\{111\}$ and $\{100\}$ planes; these planes and their intersections are typical diamond crystallographic features^{10,21,22}. The texture of the step itself is a reconstructed $\{100\}$ -(2×1) plane, extending along a $\langle 011 \rangle$ direction, and can have two principal alternating patterns, one with dimer chains oriented perpendicular and the other parallel to the lower intersection boundary; these orientations are shown on the left and right sides, respectively, of the bottom panel in Fig. 1. We note that the growth model discussed below does not limit the length of the step to an atomic size and can be applied to a macroscopic range, such as seen in scanning electron microscopy. Also, the specific step configuration chosen for the present study, a $\{100\}$ facet between $\{111\}$ terraces shown in Fig. 1, is only one of several observed configurations⁵.

The growth of the $\{100\}$ -(2×1) plane of the step at distances remote from the plane intersection, for either perpendicular or parallel orientation, is assumed to follow the mechanism established in our previous study¹⁶: insertion of CH_2 groups into a dimer, initiated either by methyl²³ or acetylene¹⁷ chemisorption, and migration of the formed CH_2 bridge sites along the directions of the original dimer chains. As no viable elementary-reaction mechanism has been proposed for the growth on $\{111\}$ planes, we assumed that reactions taking place at these surfaces—abstraction of surface hydrogen, combination of surface radicals with gaseous hydrogen atoms and methyl radicals, and their thermal desorption—do not affect the step growth. Thus we concentrate on the specific reactions occurring at the plane intersection, and how they support the stepped growth.

We first consider the dimer-chain orientation perpendicular to the plane intersection. As mentioned above, the dimer sites convert to CH_2 bridge sites with the simultaneous migration of the latter along the chains. When such a CH_2 bridge site appears at the plane intersection, it migrates further as shown in Fig. 2. Calculations show that this near-intersection migration is similar to the in-plane migration of CH_2 (ref. 16): under conditions typical of diamond CVD, the reaction rates in both forward and reverse directions are much faster than the reactions of gaseous precursors with surface sites, implying that these surface

FIG. 1 Top, schematic diagram of the model step. Bottom, illustrations of the perpendicular (left) and parallel (right) orientations of the step surface. Hatched spheres, carbon atoms of bulk diamond; black spheres, top-layer carbon atoms of the $\{111\}$ plane; grey spheres, top-layer carbon atoms of the $\{100\}$ plane; and white spheres, hydrogen atoms.

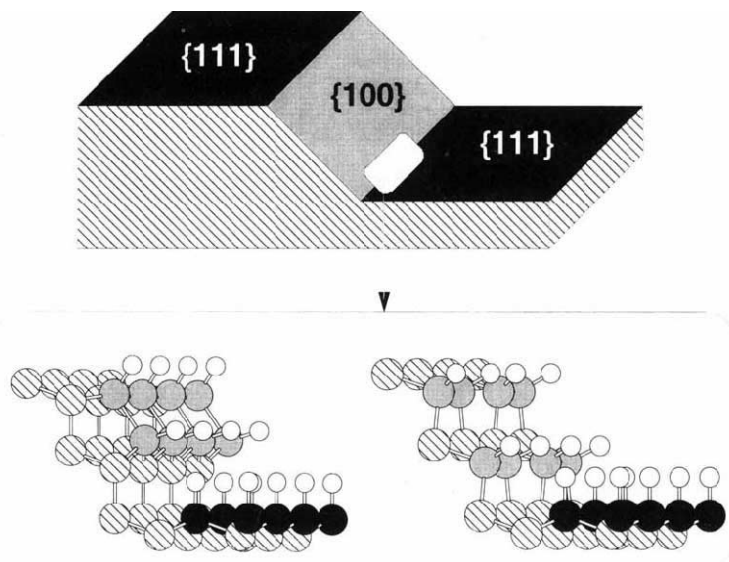
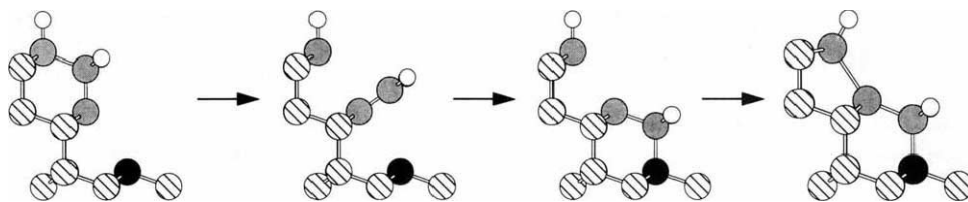


FIG. 2 CH₂ migration at the bottom of a dimer chain oriented perpendicularly to the lower plane intersection. The forward/reverse rate constants at 1,200 K calculated for the reactions depicted from left to right are $1.5 \times 10^7/6.5 \times 10^9$, $1.2 \times 10^{11}/1.7 \times 10^8$ and $7.5 \times 10^{11}/1.2 \times 10^{11} \text{ s}^{-1}$, respectively, with the overall equilibrium constant thus equal to 10.



reactions maintain a state of partial equilibrium. Furthermore, the present calculations indicate that the overall equilibrium of the reaction sequence shown in Fig. 2 is substantially shifted to the right, in the direction of CH₂ migration toward the intersection. The direction and magnitude of this shift results from the rigidity of the top-layer carbon atom of the {111} plane, that is, of the carbon atom marked in solid black in Fig. 2. In fact, this atom is constrained to such a degree that it cannot form a bond with the adjacent carbon atom of the {100} plane even when both have unfilled vacancies, as illustrated by the structure on the extreme left of Fig. 2.

The above result indicates that the reaction sequence shown in Fig. 2 is essentially irreversible under the conditions typical of diamond CVD. Once it takes place, it "pulls" the bridge sites toward it. Indeed, consider a perpendicularly-oriented chain with all but one of the CH₂ groups inserted. The equilibrium constant

of an overall CH₂ migration process, that is, the migration of the void site from the bottom of the step to its top, is exactly the same as that of the reaction sequence in Fig. 2. This is because the migration of CH₂ between internal sites, locally similar to one another, is equally probable; stated differently, the equilibrium constant for the individual CH₂ migration within the chain is unity. So at a temperature of 1,200 K, typical of diamond CVD, the probability of the void site being at the top of the step is roughly tenfold greater than it being at the bottom. In other words, the perpendicularly-oriented dimer chains are filled from the bottom of the step up to its top, which is consistent with the stepped growth model.

Thus the dimer with the structure shown at the extreme right of Fig. 2 is converted to a bridge site (by either insertion of gaseous CH₂ or downward migration of surface CH₂) and then the dimer next to it is converted to a CH₂ bridge, and so on. After the dimer chains of the step surface are filled with CH₂ groups and new dimers of the next layer are created, they are oriented parallel to the lower plane boundary, that is, they form a configuration shown on the right-hand side of the bottom panel in Fig. 1. As mentioned earlier, the growth away from the intersection proceeds by the insertion of CH₂ groups into dimers, and subsequent migration of the resulting CH₂ bridge sites. One question remains to be answered: the nature of growth just next to the lower plane intersection. The reaction pathways identified by our calculations to be most plausible for this process are shown in Fig. 3. They include both the possibility of growth by the addition of methyl to dimer radicals, shown as the reaction sequence on the left of Fig. 3, and by the addition of acetylene molecules to dimer biradicals, shown on the right of Fig. 3. The elementary reactions involved in these processes—hydrogen abstractions, methyl and acetylene additions, β -scissions and hydrogen migrations—are analogous to respective in-plane reactions^{16,17}. Also, calculations showed that kinetic and thermodynamic parameters of the near-intersection reactions (Fig. 3) were very similar to, yet not exactly the same as, those of the in-plane reactions. The higher rigidity and congestion of the intersection sites result in slower hydrocarbon addition to surface radicals and less thermodynamically stable adducts; however the numerical differences between near-intersection and in-plane parameters are typically small, no larger than one order of magnitude, and thus do not change the main kinetic character of the growth mechanism. The last reaction in the sequence of Fig. 3 is the same as the penultimate reaction in the sequence shown in Fig. 2. The equilibrium constant of this reaction is calculated to be 6.7×10^2 , showing that it is largely shifted toward the final structure (a CH₂ group bridging over the intersection), thus providing the 'driving force' for filling the dimer chain oriented parallel to the plane intersection.

Filling the parallel-oriented dimer plane with the CH₂ groups forms a perpendicularly-oriented dimer plane, and so on. The sequential repetition of these growth patterns constitutes propagation from left to right of the step shown on the top panel of Fig. 1. The particular character of this step propagation is determined by the competition between reactions comprising the process. If the CH₂ surface migration dominates the insertion of CH₂ into the edge dimers directly from the gas phase, the

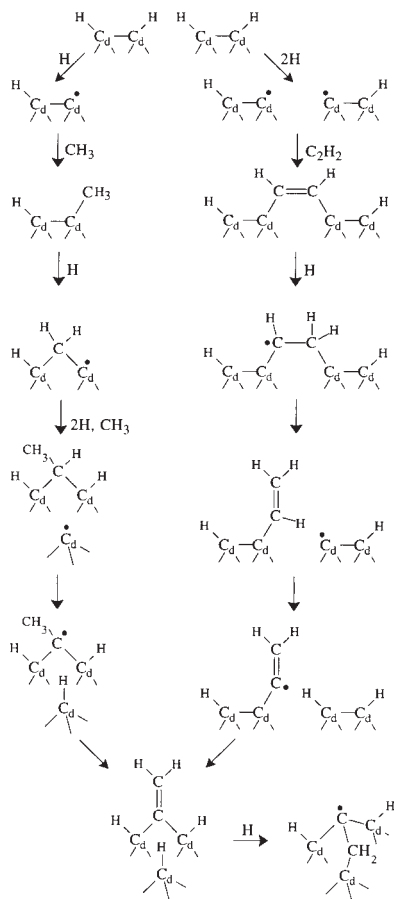


FIG. 3 Surface reactions for growth at the dimer chain located next to, and oriented parallel with, the lower plane intersection. For clarity, some reaction steps combine several elementary reactions. C_d denotes a carbon atom of the diamond lattice.

step propagates as a smooth {100} facet. If the gas-phase incorporation of CH₂ dominates the CH₂ surface migration, then the deposition proceeds as a {111} monolayer growth, filling the {111} terrace faster than the {100} facet of the step. The latter regime represents another type of the step growth, a {111} facet between {100} terraces. Many other cases can occur between these two extremes.

The two key elements of this model—the migration of chemisorbed CH₂ groups along dimer chains of diamond {100} surfaces and the high thermodynamic stability of the CH₂ bridge sites formed at the plane intersection—conform in a general

sense to the scheme of stepped growth, surface diffusion of the growth species and its lower mobility at the step. The difference in the case of diamond is that the migrating species is not a gaseous precursor itself but its surface derivative, and that the migration process is not a physical diffusion but a chemical reaction of a covalently-bonded group. The kinetic competition between the CH₂ surface migration and the incorporation of CH₂ into surface dimers directly from the gas phase determines the morphological character of the growth, and hence understanding and quantifying this competition should provide guidance for controlled CVD synthesis of high-quality diamond. □

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Carbon-cycle imbalances in the Sargasso Sea

Anthony F. Michaels*, Nicholas R. Bates*, Ken O. Buesseler†, Craig A. Carlson‡ & Anthony H. Knap*

* Bermuda Biological Station for Research, Ferry Reach GE01, Bermuda

† Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543, USA

‡ Horn Point Environmental Laboratory, University of Maryland, Cambridge, Maryland 21613, USA

THE net exchange of carbon dioxide between the atmosphere and the ocean, and thus the nature of the oceanic carbon sink, is dominated by the seasonal dynamics of carbon cycling in the upper ocean. This cycle represents a balance between abiotic and biotic carbon transport into, and export out of, the ocean's upper layer. Here we report measurements of these processes made over five years in the Sargasso Sea off Bermuda, as part of the US Joint Global Ocean Flux Study (JGOFS). We find that the decrease in carbon stocks from the spring to the autumn in the upper 150 m of the ocean is three times larger than the measured sum of biotic and abiotic fluxes out of this layer. This discrepancy can be explained either by failure to account for horizontal advection of carbon or by inaccuracies in the fluxes of sinking particles as measured using sediment traps. Either the traps miss 80% of the sinking particles, or 70% of the carbon cycling is due to advection (or a combination of both processes is responsible). Sediment-trap measurements of the ²³⁴Th flux during this period suggest that most of the discrepancy may be due to inaccuracies in the trap methods, which would require a very general reassessment of existing ideas about particle export and remineralization of carbon in the oceans. If, on the other hand, advection is the main source of the discrepancy, the traditional one-dimensional (vertical) modelling of the oceanic carbon cycle cannot give a full account of carbon dynamics.

The Bermuda Atlantic Time-series Study (BATS; part of the US Joint Global Ocean Flux Study (JGOFS)) has sampled the

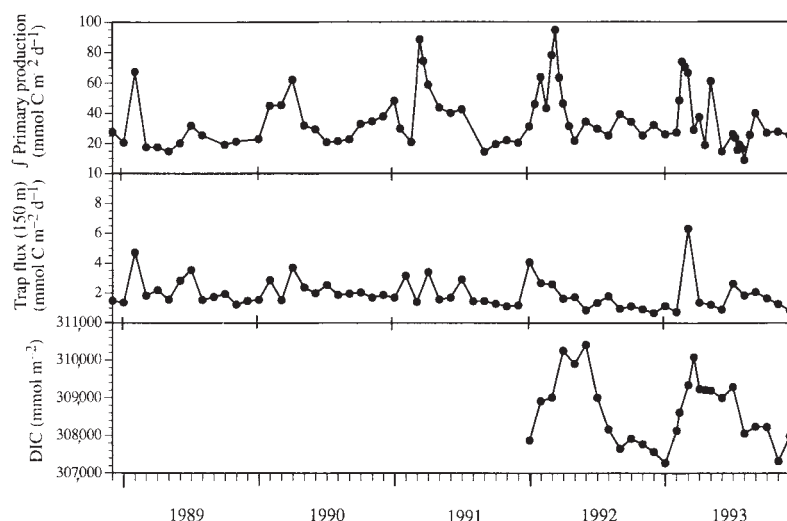
Sargasso Sea (31° 50' N, 64° 10' W) biweekly to monthly since October 1988. The programme includes measurements of most components of the carbon cycle using modern high-precision techniques^{1,2}. The shallow surface-tethered (SS-T) sediment-trap protocols³ are nearly identical to those used in most other biogeochemical investigations of the upper ocean, including the JGOFS North Atlantic Bloom Experiment, the 10-yr VERTEX and RACER programmes and the Hawaiian Ocean Time-series.

Primary production at the BATS site exhibits a strong seasonal pattern, with significant interannual variability (Fig. 1; see legend for methods). Spring blooms result from the input of nutrients following deep winter mixing, and the strength of the bloom is related to the duration and depth of mixing^{1,4}. Sediment trap carbon fluxes at 150 m are less variable. During the first two years the production peaks appeared to be coincident with peaks in trap collection, but this pattern is inconsistent in subsequent years, calling into question the earlier conclusions of a tight linkage between production and export⁵.

The seasonal cycle of dissolved inorganic carbon (DIC) shows that integrated stocks over depths of 0–150 m are low in the autumn and highest during the spring, when deep mixing brings DIC-rich water to the surface (Fig. 1). From April to December mixed layers are less than 150 m deep, which simplifies the mass-balance calculations, and we restrict our comparisons to this 275-day period (hereafter 'period'). In 1992 and 1993, there was an average decline of 2.6 mol C m⁻² period⁻¹ in the integrated stock of DIC (Fig. 2) between April and December. This DIC drawdown occurs in the absence of measurable nutrients and is an extreme example of the DIC depletions at high (non-Redfield) ratios of C to N that are observed in the North Atlantic⁶. Such non-Redfield-ratio behaviour poses a major puzzle for our understanding of the oceanic carbon cycle and must be adequately explained to model fully the role of the oceans in the global carbon budget. Nitrogen fixation is one possible source of the nutrients to support this DIC drawdown; inputs of nitrogen from atmospheric deposition are too small to produce this signal⁷. The magnitude of the seasonal carbon drawdown agrees with earlier geochemical estimates of new production at this site^{8,12}.

The integrated (0–150 m) stock of dissolved organic carbon (DOC) declined from April to November by 0.57 mol m⁻²

FIG. 1 Integrated (0–140 m) primary production ($\text{mmol m}^{-2} \text{d}^{-1}$), sediment-trap organic carbon flux at 150 m ($\text{mmol m}^{-2} \text{d}^{-1}$) and the 0–150-m integrated stock of dissolved inorganic carbon (mmol m^{-2}). Inorganic carbon fluxes were only measured in the first three years and they averaged 22% of the organic carbon flux. The DIC measurements were made using a high-precision coulometric technique¹⁵; the high-quality DIC time series is restricted to 1992 and 1993. The sediment traps are cylindrical, surface-tethered MultiPITS³⁴, deployed for 3–4 days each month. The traps were filled with a brine solution. Although these brines are known to affect collection rates, this method (using brines of 50 p.p.t. above ambient salinity) is the same as was used to collect most of the existing particle flux data sets from the upper ocean.



period⁻¹ (Fig. 2)¹³. The stocks of particulate organic carbon (POC) are small (0.3 mol m^{-2}) and relatively constant. The total loss of carbon is $3.17 \text{ mol C m}^{-2} \text{ period}^{-1}$ (Table 1). This loss must be caused by a net export of carbon from the sinking of large particles¹⁴, air-sea exchange of CO_2 (ref. 15), vertical mixing, downwelling, horizontal advection and vertical migration by zooplankton¹⁶. The average daily loss is $11.5 \text{ mmol C m}^{-2} \text{ d}^{-1}$, which is 38% of the average rate of primary production.

For closure of the (one-dimensional) carbon cycle at the BATS station, we start by considering the vertical fluxes from April to December. The SS-T trap organic and inorganic carbon fluxes averaged 0.45 and $0.1 \text{ mol C m}^{-2} \text{ period}^{-1}$ respectively (Table 1). The flux of CO_2 to the atmosphere¹⁵ is estimated at 0.05 – $0.09 \text{ mol C m}^{-2} \text{ period}^{-1}$. The mean gradient of total carbon at 150 m ^{13,15} is $0.1 \text{ mmol C m}^{-4}$ (increasing concentration with depth) and we calculate a net flux into the euphotic zone by mixing of $0.24 \text{ mol m}^{-2} \text{ period}^{-1}$. Published measurements from the Sargasso Sea of the flux of carbon by the migration of zooplankton¹⁷ yield fluxes at 150 m of 0.23 – $0.73 \text{ mmol C m}^{-2} \text{ d}^{-1}$, equal to 0.06 – $0.2 \text{ mol C m}^{-2} \text{ period}^{-1}$. Downwelling rates near Bermuda are $\sim 4 \text{ cm d}^{-1}$ (ref. 18). If the downwelling at 150 m (mean DIC, $2,080 \mu\text{mol kg}^{-1}$) is balanced by lateral inputs of surface waters (mean DIC, $2,035 \mu\text{mol kg}^{-1}$), the calculated net flux is $0.50 \text{ mol C m}^{-2} \text{ period}^{-1}$. The sum of all of these vertical processes is a net export of $1.01 \text{ mol C m}^{-2} \text{ period}^{-1}$. The residual discrepancy is $\sim 2.2 \text{ mol C m}^{-2} \text{ period}^{-1}$.

Clearly, the carbon system at BATS cannot be balanced within a factor of three with these vertical rates. Either some of the data are incorrect or horizontal advection determines the observed patterns. The uncertainties in DIC and DOC are small and the annual repetition of the pattern indicates that it is robust. Surface DIC measurements since 1983 show the same seasonal pattern as in 1992 and 1993 (ref. 19). The fluxes from vertical mixing increase the carbon stock in the euphotic zone. Most direct measurements of the carbon flux by zooplankton in oligotrophic environments yield similarly low rates¹⁷. The downwelling estimates are conservative; refinements will probably lower the flux (Table 1). There remain two possible explanations for the discrepancies in the carbon budget: either the SS-T traps are inaccurate tools for determining the export of carbon on sinking particles, or the carbon cycle at BATS is largely determined by horizontal advection.

There is no unambiguous method for determining the accuracy of sediment traps in the field. Acceptance has come through the consistency of trap data with existing paradigms^{20,21}: fluxes were higher in areas, and during seasons, of higher productivity, fluxes declined with depth, they were more consistent in deep water, and repeat observations in the same area yielded similar

values. Early comparisons of the scavenging of ^{234}Th agreed with collection by SS-T traps^{22,23}. As a result, SS-T trap data have been used widely in upper-ocean carbon models and descriptions of the global carbon cycle. However, recent data again raise questions of the accuracy of SS-T traps. Drifting cylinders and cones may differ 40-fold²⁴. Comparisons of ^{234}Th scavenging and ^{234}Th fluxes in traps have shown large disparities^{25,26}.

SS-T traps at the BATS site could be inaccurate if they miss large episodic flux events or are biased in collecting sinking particles. The BATS traps sample three days in each month for a total of 27 days (nine 3-day samplings) of the 275 days between April and December. For the years 1989–1993, there is a 95% probability that one or more of the trap deployments would have co-occurred with a high-flux event if 30 or more of these hypothesized events had occurred over the 5 years. To account for the discrepancy of $2.2 \text{ mol C m}^{-2} \text{ period}^{-1}$, the average daily flux in each proposed event would have to be $122 \text{ mmol C m}^{-2} \text{ d}^{-1}$. This rate exceeds the highest measurements of primary production at BATS and the 3-day sum at this rate exceeds the entire stock of POC. Thus, the difference between the carbon deficit and trap fluxes cannot be explained by stochastic processes.

Trap biases take three forms: swimmer biases, solubilization of particulate matter in the trap, and hydrodynamical biases. The removal of swimmers (zooplankton that actively enter the trap) is difficult and imperfect removal will increase the apparent flux (a bias in the wrong direction). In several studies, SS-T trap fluxes were multiplied by 2.23 to correct for a presumed solubilization of particles inside the traps^{27,28}. However, the screened, live traps used to estimate the dissolved organic matter solubilization²⁹ contained living zooplankton³⁰. Experiments with swimmer-exclusion traps show a solubilization of only 7% of the POC³¹. Dissolution of CaCO_3 in the trap is also unlikely as a large CaCO_3 flux would change the alkalinity–salinity relationships at the surface, and these deviations were not observed¹⁵. Hydrodynamic biases are known, but are still poorly understood^{20,32}. The level of brine/preservative in a trap affects collection (W. Gardner, unpublished data) and many of the recommended changes to trap protocols involve restricting the brine solutions to the lower part of the trap²⁰. We fill the traps with brine (50 p.p.t. above ambient salinity) before deployment, the same protocol as has been used in many upper-ocean trapping programmes^{27,33–35}. Hydrodynamic biases are a likely source of trap errors and the interaction between traps, the brine solutions and the ambient flow field must be resolved. However, this resolution requires the development of an unambiguous method for determining the absolute accuracy of SS-T traps in the field.

TABLE 1 Carbon balance at the Bermuda Atlantic Time-series Study (BATS) site between 1 April and 31 December

Process	Mean daily rate (mmol C m ⁻² d ⁻¹)	Integrated rate (mol C m ⁻² period ⁻¹)	Per cent total carbon drawdown
Changes in suspended carbon stocks			
ΔDIC	-9.4	-2.60	82.0
ΔDOC	-2.1	-0.57	18.0
ΔPOC	~0	~0	0.0
ΔTotal carbon	-11.5	-3.17	100.0
Fluxes in (positive) and out (negative) of the 0–150 m layer			
Sinking particles	-2.0	-0.55	17.4
Gas exchange	-0.3	-0.07	2.2
Vertical mixing	0.9	0.24	-7.6
Zooplankton migration	-0.5	-0.13	4.1
Downwelling	-1.8	-0.50	15.8
Balance of fluxes	-3.7	-1.01	31.9

Stocks are integrated over the upper 150 m. DIC data are shown from 1992 and 1993 (Fig. 1)¹⁵; DOC data are from 1993 (Fig. 2)¹³. Particle flux data are the sum of the organic carbon flux (1988–1993) and inorganic carbon fluxes (1988–1991) from the 150-m trap. Gas exchange is calculated using a $p\text{CO}_2$ calculated from 1992–1993 DIC and alkalinity¹⁵ and measured wind speeds. The average of the flux is calculated with two different algorithms for the transfer coefficient¹⁵. The magnitude of the vertical gradient of DIC, which increases with depth, was larger than the DOC or POC gradients, which decreased with depth, yielding a net increase in total carbon (DIC + DOC + POC) with depth. Vertical mixing was calculated with the gradient in total carbon and an eddy diffusivity of $1 \times 10^{-4} \text{ m}^2 \text{ s}^{-1}$ (indicated by various modelling studies⁹). Carbon flux due to zooplankton vertical migration is taken as the midpoint in the range of all values for the Sargasso Sea¹⁷; rates from open ocean sites were lower than this value. Downwelling flux assumes that the low-DIC surface waters replace all of the downwelling. This maximizes the gradient and the flux; the true gradient is probably somewhat lower.

The most direct assessments of SS-T trap accuracy have used comparisons of ^{234}Th -scavenging profiles (used to predict ^{234}Th export on particles) with the ^{234}Th collected by SS-T traps^{22,23,25}. In one third of the studies, the predicted and observed fluxes disagree threefold or more²⁵, with both under- and over-collection. BATS SS-T traps collected half as much ^{234}Th as predicted, with a fourfold discrepancy in June (Fig. 2). The seasonal pattern in the predicted ^{234}Th export is similar to the DIC removal and different from the monotonic trap collections. The consistency of POC-to- ^{234}Th relationships is unknown, particularly if traps sort the particles. The POC-to- ^{234}Th ratios in the BATS traps range from 2.1 to 9.6 $\mu\text{mol C per d.p.m.}$ Assuming this ratio is representative of true sinking particles, we calculate a POC flux of 0.4 to 1.9 $\text{mol C m}^{-2} \text{ period}^{-1}$ from the predicted ^{234}Th export. Inclusion of PIC would increase these fluxes by another 20%. This range of rates could explain up to 85% of the carbon discrepancy.

The other possible explanation for the carbon imbalance is horizontal advection, the seasonal movement of different water masses past the BATS station. The mean flow near Bermuda is small and predominantly from the northeast as part of the Gulf Stream recirculation³⁶. For advection to mimic the seasonal DIC pattern requires the transport of progressively lower DIC water masses over distances of 300–1,000 km. DIC varied by less than 12 $\mu\text{mol kg}^{-1}$ on seasonal 600-km north–south transects and concentrations were higher north of the island (unpublished data). Much of the DIC drawdown occurs over a few months (Fig. 1), a timescale on which local effects probably dominate over net advection. Eddies are apparent in the time series of salinity at BATS, but DIC variations are not strongly related to the salinity fluctuations. Thus, horizontal advection probably cannot explain the entire DIC pattern, but it cannot be ruled

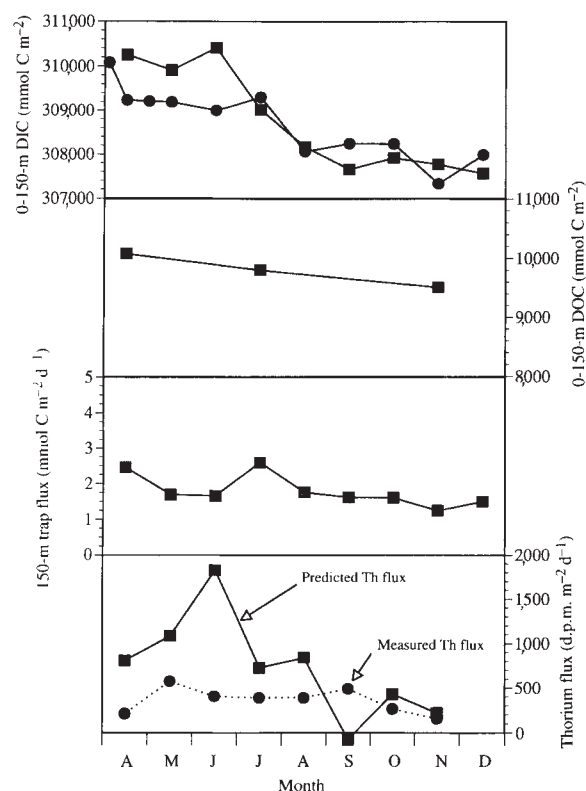


FIG. 2 Summaries of the changes in the stocks of carbon and the rates of export of carbon and ^{234}Th between April and December. The integrated (0–150 m) DIC values are the biweekly to monthly values for 1992 (squares) and 1993 (circles). The integrated (0–150 m) DOC measurements were made over two- to three-week periods in each of three months in 1992 using a high-temperature catalytic combustion technique¹³. The organic carbon flux is the average of sediment trap measurements in each month over the entire 5-year period. The predicted ^{234}Th flux is derived from comparison of ^{234}Th profiles collected in 1993 with the predicted equilibrium ^{234}Th , and the flux is calculated using a steady-state model²⁶. Three ^{234}Th profiles were collected on each cruise to allow an assessment of day-to-day variability or non-steady-state behaviour of the ^{234}Th . The measured ^{234}Th flux is from sediment trap samples collected during 1993 on the same cruises as the profiles.

out. Advection is the most difficult component of the carbon balance to interpret and measure.

Many disagreements about the magnitude of new production in the Sargasso Sea will be resolved if sediment traps undercollect at this site, or if the biogeochemistry is controlled by advection^{1,2,10,12,28,37}. Although we cannot conclude from assertions of inaccuracies in BATS traps that the fluxes measured elsewhere are inaccurate, the physical environment at the BATS site is benign and the BATS methods are nearly identical to many other programmes. We suggest that data from SS-T traps must be viewed critically in every experiment and that the level of supporting data from tracers and elemental mass balances must be increased. Ultimately, to attain the goals of international ocean biogeochemistry programmes, like JGOFS, we must expand from traditional one-dimensional approaches to studies of the ocean in three-dimensions. □

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A deep earthquake aftershock sequence and implications for the rupture mechanism of deep earthquakes

Douglas A. Wiens*, Jeffrey J. McGuire*, Patrick J. Shore*, Michael G. Bevis†, Kitlone Draunidalo‡, Gajendra Prasad‡ & Saimone P. Helu§

* Department of Earth and Planetary Sciences, Washington University, St Louis, Missouri 63130, USA

† Hawaii Institute of Geophysics and Planetology, University of Hawaii, Honolulu, Hawaii 96822, USA

‡ Fiji Mineral Resources Department, Suva, Fiji

§ Tonga Ministry of Lands, Survey, and Natural Resources, Nuku'alofa, Tonga

A distinguishing characteristic of deep earthquakes has been the absence of observable aftershock sequences^{1,2}. Here we report the first extensive deep-earthquake aftershock sequence to be observed; it was recorded by an array of eight broadband seismographs following the 9 March 1994 deep Tonga earthquake. The aftershocks show a power-law decay with time following the main shock, as is typical of shallow events. Most of the well located aftershocks are concentrated along a steeply dipping plane consistent with one of the nodal planes of the main-shock mechanism and the mechanisms of three large aftershocks. Assuming these aftershocks denote the main-shock rupture area, they define a 50×65 km fault plane extending across the entire width of the active seismic zone and into the surrounding aseismic region. The width of the aftershock zone is wider than the expected width of the metastable olivine wedge, demonstrating that either the width of the metastable olivine material exceeds previous estimates, or the aftershocks are not confined in such a wedge.

It has long been noted that an important difference between deep earthquakes (depth > 300 km) and shallow earthquakes (depth < 70 km) is that the former produce very few aftershocks. Shallow earthquakes are characterized by numerous aftershocks showing an exponential decay with time. A few such aftershock sequences have been reported for intermediate-depth events^{3,4}, but well developed aftershock sequences have not been observed for deep earthquakes. This difference in aftershock behaviour has been used as primary evidence for a difference in earthquake generating mechanisms between shallow and deep earth-

quakes^{1,2,5,6}. The lack of aftershocks for deep earthquakes has also prevented delineation of the rupture zones of large, deep earthquakes. Previous studies suggest that the few observed deep-earthquake aftershocks do not seem to cluster along the fault planes of the main shocks⁷, as aftershocks do for shallow events.

The 9 March 1994 (moment magnitude $M_w = 7.6$; depth, 564 km) earthquake in the Tonga slab was larger than any deep earthquake during the previous 20 years. This earthquake was followed by an unprecedented aftershock sequence, which exceeds the number of aftershocks of all deep earthquakes during the past 35 years (Table 1), including the recent deep Bolivia earthquake ($M_w = 8.3$)⁸, the largest deep earthquake ever recorded. In addition, the deep Tonga earthquake occurred beneath an array of portable digital broadband seismographs installed in Tonga, Fiji and Niue Island (Fig. 1) which enables detailed study of the locations and focal mechanisms of the after-

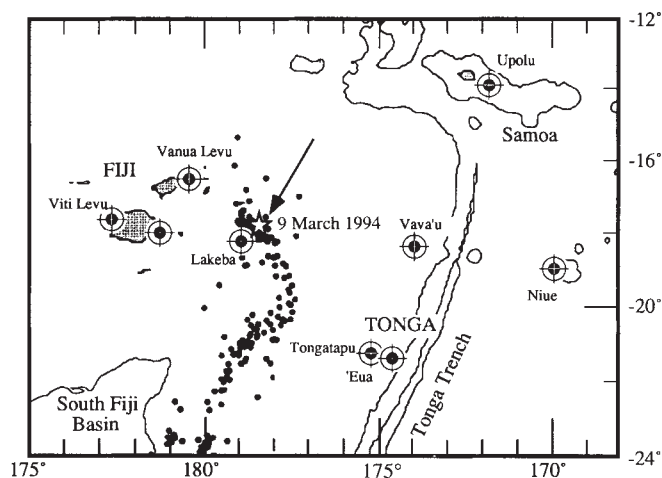


FIG 1. Map of the southwest Pacific showing the location of the 9 March 1994 deep Tonga earthquake (star) and the locations of regional digital broadband seismic stations (large black dots with cross). All stations are part of a temporary network deployed in late 1993 except for Samoa, which is a permanent IRIS station. The 4,000-m and 8,000-m bathymetric contours are shown, along with the locations of a 2-year sample of deep earthquakes in the Tonga slab (small black dots). Seismograph locations are denoted by the island name. The arrow shows the strike of the steeply dipping fault plane of the 9 March event, as determined from the aftershock distribution.

TABLE 1 Aftershocks of large deep earthquakes, 1960–94

Date	Location	M_w	Depth	Number of aftershocks		
				$m_b \geq 5.0$	$m_b \geq 4.5$	Total
15 Aug. 1963	Peru	7.8	543	0	0	0
11 Nov. 1963	Peru	7.6	600	1	2	5
7 Oct. 1968	Izu–Bonin	7.4	457	0	1	1
31 July 1970	Columbia	8.2	653	0	0	0
30 Aug. 1970	Sea of Okhotsk	7.3	643	0	0	0
29 Sept. 1973	Sea of Japan	7.8	567	0	0	0
22 June 1982	Banda Sea	7.4	450	0	0	0
6 Mar. 1984	Izu–Bonin	7.4	457	0	1	2
9 Mar. 1994	Tonga–Fiji	7.6	564	11	40	82
9 June 1994	Bolivia	8.3	636	1	—	36

Earthquakes include all events 300–700 km deep with seismic moment $>1 \times 10^{20}$ Nm, from Abe²⁹ and the Harvard centroid moment tensor catalogue³⁰. Aftershock data is from the International Seismological Centre, Newbury, UK, except for the 1994 Tonga data which are from the monthly Preliminary Determination of Epicenters (PDE; US Geological Survey) and the regional network, and the 1994 Bolivia data which are from the weekly PDE and Myers *et al.*⁸. The total number of aftershocks is biased by different detection thresholds, but detection should be uniform at the body-wave magnitude $m_b = 5.0$ level. (M_w , moment magnitude.)

shocks. This array recorded a sequence of 82 aftershocks that extended for at least 42 days and ranged in body-wave magnitude (m_b) from 3.8 to 6.0. Many more probable aftershocks were visible on the records of the two best stations during the initial hours after the main shock.

The aftershocks show a rapid power-law decay with time (Fig. 2), as is observed for the aftershock sequences of shallow earthquakes^{9–11}. We fitted the time distribution of the aftershocks using a maximum-likelihood fit to the modified Omori relationship^{12,13} $N(t) = K(t+c)^{-p}$, where $N(t)$ is the number of earthquakes per unit time and K , c and p are empirical constants. This relation provides a good fit to the decay rate (Fig. 2), with $p = 1.0$ giving the optimum fit. This is identical to the average p value for shallow earthquake sequences^{10,11}, suggesting similarities in the aftershock generating process between deep and shallow earthquakes. But it differs from the only previously suggested p value for deep earthquakes, $p = 0.54$, obtained from

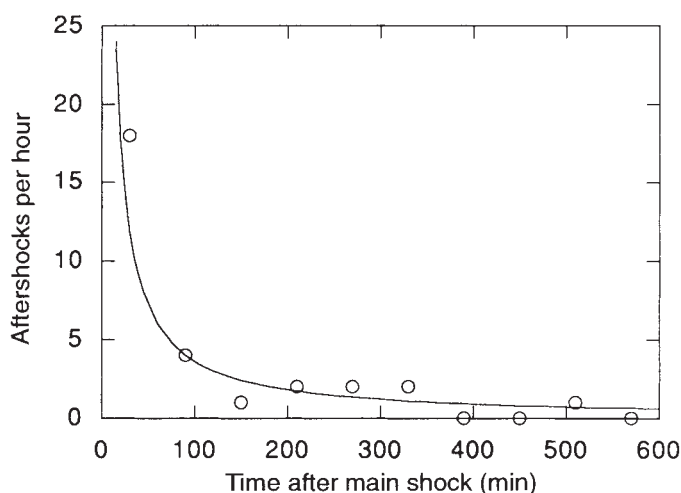


FIG. 2 Plot of the number of aftershocks per hour (open circles) as a function of time after the main shock. Also shown is a line representing the maximum-likelihood fit to the modified Omori relationship^{12,13} of the aftershock times from the first 18 days of the sequence. The aftershock decay is best fitted by a power-law decay with exponent $p = 1$, similar to aftershock sequences of shallow earthquakes.

superposition of data from many earthquakes which individually had too few aftershocks to obtain a result¹¹.

We determine the relative positions and uncertainties of the main shock and aftershocks by inverting P and S phases from the regional network along with teleseismic P, pP and PKP phases using a hypocentral decomposition algorithm¹⁴. This method uses arrival times from different earthquakes at the same station to constrain earthquake relative positions within a limited source region, thus minimizing the effect of velocity heterogeneity along the ray paths. The results are displayed and analysed using a three-dimensional graphics package which renders the 95% confidence volumes of the earthquake locations as geometric solids¹⁵.

The main shock and 14 out of 18 well located aftershocks are localized along a nearly vertical plane (Fig. 3a). Three well located aftershocks not displayed in Fig. 3a were located at least 70 km from the main shock hypocentre and seem unlikely to be part of the main-shock rupture zone. The plane that has the best least-squares fit to the locations of nine aftershocks that occurred during the first day following the main event has strike 30° , dip 68° , and the maximum distance of any of the events from the plane is 5 km.

This plane is in good agreement with a steeply dipping NNE-striking nodal plane in the focal mechanism of the main shock (Fig. 4 top). Focal mechanisms were determined by inverting the regional and teleseismic body waveforms using synthetics calculated with a reflectivity method^{16,17}. The result shows a north–south striking, steeply dipping fault plane (strike 5° , dip 70°), and is nearly identical to the Harvard centroid moment tensor solution for the main shock (G. Ekstrom and A. Dziewonski, personal communication). This plane deviates by 25° in strike from the plane determined from the aftershock locations. There is, however, considerable evidence for a change in focal mechanism as the rupture progressed, including changes in the polarity of broadband-displacement P and S waves during the rupture pulse and the results of preliminary studies of the main-shock rupture process¹⁸ (M. Kikuchi, personal communication). Also plotted in Fig. 4 (top) is a focal mechanism determined solely from P and S wave first motions; it differs by only 5° in strike and 4° in dip from the plane determined from the aftershock locations, demonstrating that the aftershock plane is consistent with the focal mechanism of the first part of the main-shock rupture.

Broadband displacement waveforms from the regional array were also inverted for the focal mechanisms of some of the larger aftershocks (Fig. 4 bottom). These events showed focal mechanisms similar to that of the main shock, with steeply dipping fault planes roughly consistent with the planar distribution of aftershocks. The strike of the steeply dipping fault plane varies from 10° to 45° , suggesting some variability in fault strike.

Within the planar region, the aftershocks concentrate near the epicentre of the main shock, but they seem to delineate a rupture zone of $\sim 50 \times 65$ km (Fig. 3b). This rupture dimension is consistent with that expected for an earthquake of this magnitude, and combined with the observed rupture duration of ~ 14 s (ref. 18) suggests a rupture velocity of ~ 2.5 km s^{-1} . If the main shock ruptured through the entire region defined by the aftershocks, this suggests a stress drop of ~ 1.6 MPa (16 bar), consistent with previous suggestions that deep earthquakes show similar stress drops to shallow earthquakes^{19,20}.

To investigate the spatial relationship between the rupture zone of the 9 March event and the Tonga deep seismic zone, we inverted for the positions of the well located aftershocks relative to teleseismically recorded earthquakes in this region using the same relocation method. The fault plane defined by the aftershocks of the 9 March event is nearly vertical and cuts across the seismically active slab, extending entirely across the thickness (~ 30 km) of the active seismic zone (Fig. 3c). Two events occur outside the active zone, as defined by the previous seismicity. These event locations are well constrained and the one to the

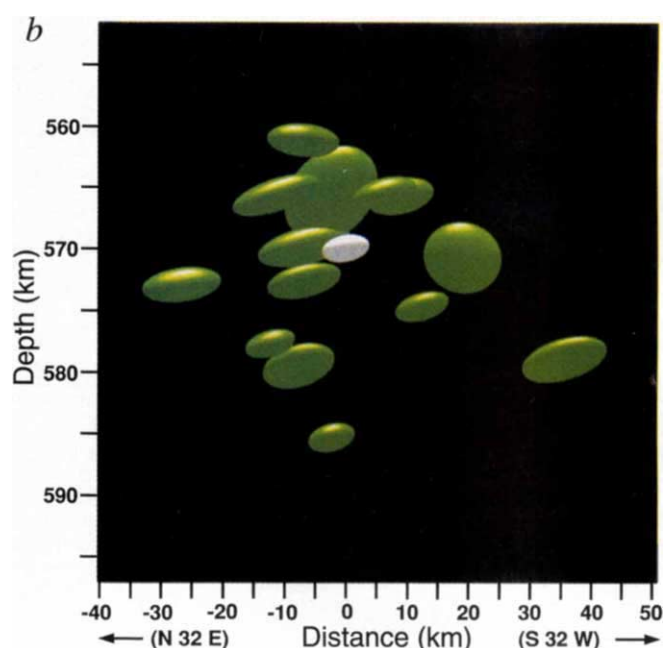
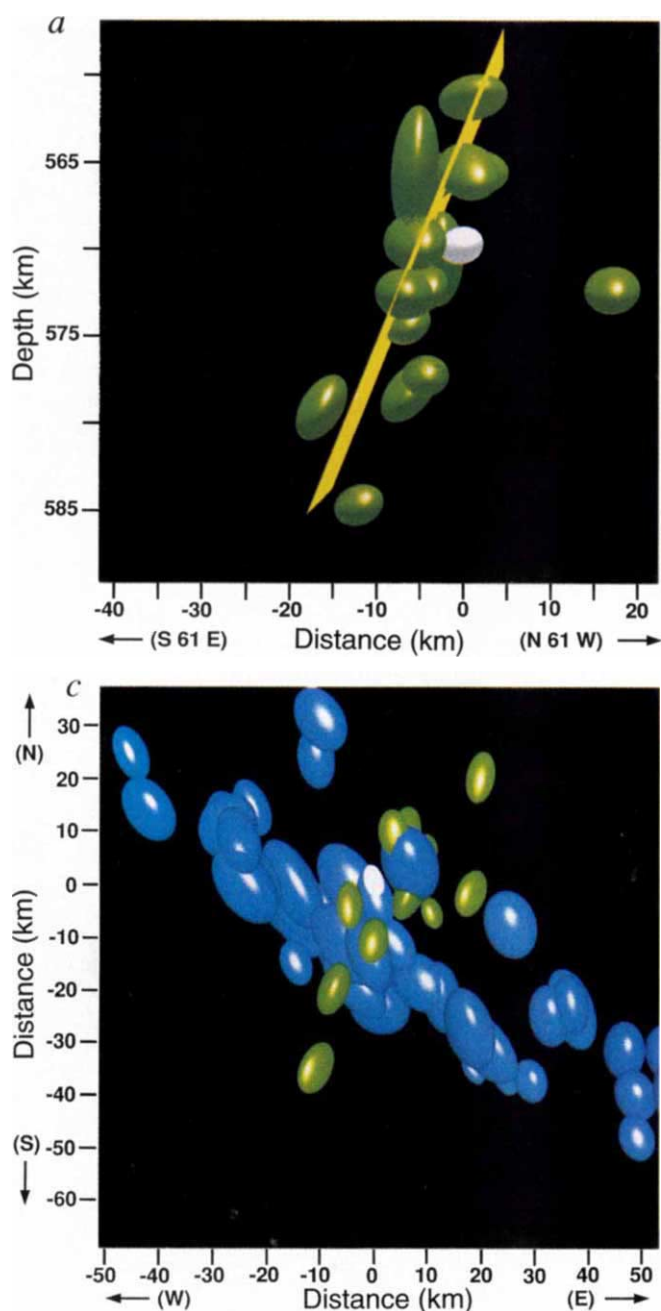
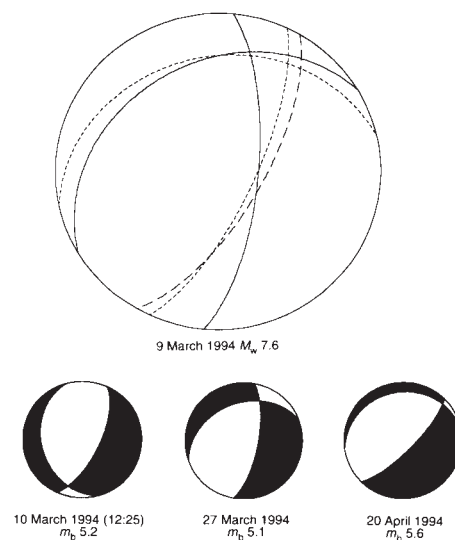


FIG. 3 Three-dimensional views of the best-located aftershocks. Each panel shows the 95% confidence ellipsoid for each earthquake determined by a hypocentral decomposition inversion¹⁴ of arrival-time data. All arrival times and associated uncertainties were picked by our research group except that teleseismic arrival times compiled by the monthly PDE bulletin were added. Only events showing 95% confidence semiaxis lengths of <8 km are plotted. All of these well located events have at least ten arrival times from our regional network as well as at least eight teleseismic arrival times. *a*, Side view of the planar configuration of aftershocks (almost edge-on to the fault plane). The main-shock epicentre is denoted by a white ellipsoid (located at 17.99° S, 178.25° W), and the aftershocks by green ellipsoids. The plane that best fits the aftershock locations is also shown (yellow). *b*, Side view perpendicular to *a* showing the spatial extent of the planar aftershock configuration. The aftershocks fill a 50×65 km planar region that probably represents the rupture zone of the main shock. *c*, Results of a joint inversion of the best-located aftershocks (green) and 1980–87 teleseismic seismicity with at least 30 recorded arrivals (blue) in the region between 17° and 19° S latitude. This is a map view (north is up) of all events between 525 and 615 km depth. Because the seismicity zone is vertical in this area, the view shows two vague linear bands of events (blue) which suggest a double seismic zone¹⁵. The events in the planar aftershock region (green) extend entirely across the seismically active portion of the slab and into the surrounding aseismic region.

FIG. 4 Focal mechanisms of the 9 March 1994 deep Tonga earthquake and larger aftershocks. Top, lower-focal-hemisphere projection of the main shock determined from first motions (short dashed lines) and from inversion of regional and teleseismic long-period three-component data (solid lines). The orientation of the plane which best fits the well located aftershocks that occurred during the first day after the main shock is also shown (long dashes). The difference between the first-motion mechanism and the long-period inversion suggests changes in the focal mechanism during the rupture, with the steeply dipping plane of the first motion mechanism showing excellent agreement with the plane determined from the alignment of aftershocks. Bottom, source mechanisms determined for the larger aftershocks from inversion of regional three-component data. All the mechanisms display steeply dipping north- to northeast-striking fault planes consistent with the main-shock focal mechanism and the alignment of aftershocks, but with some variability indicating heterogeneity in fault orientation. The 27 March 1994 event, located outside the active slab as defined by the background seismicity (Fig. 3c), shows a focal mechanism nearly identical to that of the main shock.



north, on 27 March, shows a focal mechanism similar to that of the main shock (Fig. 4 bottom). This suggests that the main-shock rupture may have propagated beyond the limits of the background seismicity in this region, or at least triggered aftershocks within the normally aseismic region surrounding the seismically active slab.

Recent research suggests that deep earthquakes may be caused by transformational faulting, as material within a wedge of metastable olivine^{21,23} is transformed to a spinel structure^{5,6,24,25}. Because the transformation is temperature-activated, deep earthquakes should occur preferentially in the warmer, outer regions of the metastable wedge, possibly forming a double seismic zone^{15,26}. The background seismicity near the 9 March event, when viewed in cross-section, shows two rather indistinct vertical alignments of earthquakes along the edges of the slab that may represent such a double seismic zone (blue ellipses in Fig. 3c).

The zone of aftershocks of the 9 March event cuts across the entire zone of seismicity and some distance into the surrounding aseismic region, extending for at least 55 km in a direction perpendicular to slab strike. Although it may be possible that the main-shock rupture did not extend all the way to the outer aftershocks, the two aftershocks unmistakably initiated outside the normal zone of seismicity. The 55 km width of the aftershock zone exceeds both the width of the background seismicity in the slab (Fig. 3c) and current estimates of the width of the metastable wedge^{15,27} based on slab thermal models²⁸ and the kinetics of the olivine-spinel reaction^{21,23} (~20 km at this depth in the Tonga slab). We propose that either the metastable olivine wedge is much wider than previously thought, because of deficiencies in the thermal models or kinetic calculations, or that the aftershocks are not confined within such a wedge. The first option seems unlikely, as it requires an explanation for the confinement of the background seismicity to a zone much narrower than the metastable wedge. If the aftershocks are occurring outside the zone of metastable olivine, then a mechanism for their occurrence other than transformational faulting must be sought. □

Combined spatial and temporal imaging of brain activity during visual selective attention in humans

H. J. Heinze^{*†}, G. R. Mangun^{†‡}, W. Burchert[§],
H. Hinrichs^{*}, M. Scholz^{*}, T. F. Münte^{||}, A. Göss^{||},
M. Scherg^{||}, S. Johannes^{||}, H. Hundeshagen[§],
M. S. Gazzaniga[†] & S. A. Hillyard[#]

^{*} Department of Clinical Neurophysiology, Otto-v-Guericke University, D-39120 Magdeburg, Germany

[†] Department of Psychology and Center for Neuroscience, University of California at Davis, Davis, California 95616, USA

[§] Department of Nuclear Medicine, Medical School of Hannover, 3000 Hannover, Germany

^{||} Department of Neurology, Medical School of Hannover, 3000 Hannover, Germany

[#] Department of Neurology, University of Heidelberg, Heidelberg, Germany

[#] Department of Neurosciences, University of California at San Diego, La Jolla, California 92093, USA

VISUAL-SPATIAL attention is an essential brain function that enables us to select and preferentially process high priority information in the visual fields^{1,2}. Several brain areas have been shown to participate in the control of spatial attention in humans³⁻⁵, but little is known about the underlying selection mechanisms. Non-invasive scalp recordings of event-related potentials (e.r.p.s) in humans have shown that attended visual stimuli are preferentially selected as early as 80–90 ms after stimulus onset^{6,7}, but current e.r.p. methods do not permit a precise localization of the participating cortical areas. In this study we combined neuroimaging (positron emission tomography) with e.r.p. recording in order to describe both the cortical anatomy and time course of attentional selection processes. Together these methods showed that visual inputs from attended locations receive enhanced processing in the extrastriate cortex (fusiform gyrus) at 80–130 ms after stimulus onset. These findings reinforce early selection models of attention⁸⁻¹⁰.

In our spatial attention task, healthy young subjects ($N=10$) attended selectively to the right or left half of bilateral stimulus arrays that were flashed in rapid sequence (Fig. 1, top). Attention was directed covertly while the subjects' gaze remained fixated on a central dot; fixation was verified with both electro-oculographic and high-resolution infrared video methods. Changes in regional cerebral blood flow (activation) as a function of selective attention were measured using the positron emission tomography (PET) $H_2^{15}O$ method. These activations were first visualized as difference images derived by subtracting the images obtained during passive viewing from those obtained in the attend-left or the attend-right conditions (Fig. 1). Focused attention produced significant PET activation in the hemisphere contralateral to the attended visual hemifield (Table 1). Specifically, attending to the left produced a significant activation in the right posterior fusiform gyrus of extrastriate visual cortex (Fig. 1, left column), whereas attending to the right produced activation in the left fusiform gyrus (Fig. 1, right column).

Significant activations were also obtained in the right anterior cingulate gyrus, and left superior frontal gyrus for the attend-left minus passive PET images (Table 1). Significant activations in the thalamus reached statistical significance only for the attend-right minus passive subtraction (Fig. 1). No significant attention-related activations were obtained in the striate cortex or parietal cortex in this task.

† To whom correspondence should be addressed.

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TABLE 1 Significant changes in regional cerebral blood flow (activation) as indicated by PET

Types of difference image	Talairach coordinates (mm)			Brain structure	Significance level (P)
	M/L	A/P	S/I		
L-P	10	-1	58	Superior frontal gyrus, left	0.02
	-3	14	34	Cingulate gyrus, anterior right	0.02
	-28	-73	-14	Fusiform gyrus, right	0.02
R-P	1	-18	0	Thalamus	0.01
	27	-77	-14	Fusiform gyrus, left	0.01

The results of two types of subtraction images were investigated using the procedure in ref. 27 (attend-left minus passive = L-P; attend-right minus passive = R-P). For each, the three-dimensional locations of the centre of the activated brain regions is indicated in coordinates of the Talairach and Tournoux atlas²⁸. M/L, Medial-lateral; A/P, Anterior-posterior; S/I, Superior-inferior. The magnitude of the activations varied from 4–6 per cent. The sample variance of the difference images estimated over all subjects was roughly constant over the whole volume, thus allowing the *t*-statistics to be calculated with a fixed standard deviation independent of localization²⁷. A three-dimensional Hanning filter (FWHM = 18.8 mm) was applied to overcome residual anatomical variability. The resulting reduction in spatial resolution is accounted for in the procedure. Weak activations that were not statistically significant were noted for the attend-left minus passive subtraction in the thalamus ($P=0.61$), and in the region of the left and right insula and claustrum ($P=0.48$ and $P=0.53$, respectively). Nonsignificant activations noted in the attend-right minus passive subtraction were in the anterior cingulate gyrus ($P=0.34$) and in the regions of the right insula and claustrum ($P=0.16$). Only increases in regional blood flow are described above and in the table: significant decreases in blood flow were observed in inferior-medial regions of the prefrontal cortex in the medial frontal gyrus and the gyrus rectus (Brodmann's areas 10 and 11) for both attend-left minus passive and attend-right minus passive subtractions (both $P<0.01$).

The e.r.ps were recorded in a separate session from the same subjects performing the identical spatial attention task. The early positive P1 component (80–130 ms after stimulus onset) was of significantly greater amplitude over the occipital scalp regions contralateral to the attended hemifield (Fig. 2a), replicating previous reports¹¹. Iso-voltage contour maps showed the P1 attention effect to be maximal over the lateral occipital cortex (Fig. 2a). There were no significant attention-related changes in the e.r.p. waveform during the earlier time period from 50–80 ms latency that reportedly corresponds to activity in the primary visual (striate) cortex ($P>0.5$)^{7,12}.

To rule out the possibility that these attention-related PET activations or e.r.p. differences were the result of nonspecific changes in brain activity between conditions of directed attention versus passive viewing, a second type of difference image was calculated by subtracting the attend-right from the attend-left condition. Such a subtraction compares two selective attention conditions in which the overall level of behavioural arousal was equivalent. In this subtraction, only the PET activations in the fusiform gyrus remained statistically significant. Similarly, the attend-left minus attend-right subtraction for the e.r.ps showed significant ($P<0.01$) effects of spatial attention in the 80–130 ms time period, with a maximum amplitude over the lateral occipital cortex (Fig. 2b, right).

To examine whether the attention-related neural activity in the fusiform gyrus shown by PET could have produced the recorded e.r.p. voltage patterns on the scalp, we modelled the neural generators of the e.r.p. activity as discrete dipolar sources¹³. To model the attend-left minus right e.r.p. attention effect (Fig. 2b, right), a dipole was placed in each hemisphere at the centre of the fusiform gyrus PET activation, and the dipole orientations were allowed to change to minimize the difference between scalp fields predicted by the forward solution and the recorded e.r.ps.

FIG. 1 Brain regions activated during spatial selective attention. The stimulus array is depicted at top, with the direction of covert attention indicated by the 'spotlight' in broken lines. Subtracted PET images (right hemisphere on right side) for attend-left minus passive (left column) and attend-right minus passive (right column) conditions are overlaid onto the corresponding section from the Talairach Atlas²⁸. Significant activations as a function of spatial attention were localized to the fusiform gyrus in the hemisphere contralateral to the attended visual hemifield (top row images), in the thalamus (second row), and in the anterior cingulate gyrus (bottom row). Scale is per cent maximal activation. Subjects attended to either the left or right half of the flashed (100 ms duration) bilateral stimulus arrays for two blocks each and passively viewed the stimuli for two blocks. The task was to press a button when the two symbols in the attended half-field were identical; these 'targets' occurred on 25% of the trials. Stimulus-onset-asynchronies varied from 250–550 ms. Symbols (each 1.9×1.4 degrees) were located 6.0 and 11.0 degrees eccentric (to centre). Mean target detection accuracy was 57.3%, and mean reaction time was 548 ms with false alarms below 10%. Sixty seconds before injection the subjects fixated and the stimuli began. Twenty seconds before injection the subjects were instructed to begin attending and performing the task. A bolus injection of 10 ml saline with 50 mCi of ^{15}O -labelled water (half-life 123 s) was injected via intravenous catheter in the right arm. Using a SIEMENS ECAT 951/31 scanner, data was acquired 20–60 s after injection. Head position was monitored using ^{68}Ge scalp markers. The anterior commissure/posterior commissure line was identified in the subjects' PET-images²⁹ and verified with MRI scans. The PET scans were reoriented and then rescaled into stereotactic space²⁸ and global normalization was performed^{25,30}. The two scans per attention condition were averaged together for each subject and filtered. Difference images were calculated and then averaged over the 10 subjects. Because tissue radiation counts are roughly linear with cerebral blood flow³⁰ the effects were considered to reflect cerebral blood flow. PET activity was also measured in each subject in spherical regions of interest (18.8 mm diameter) centred on the coordinates of maximal significant activations²⁷ for analysis with repeated-measures ANOVA. A significant interaction ($F[1, 9]=41.5$, $P<0.001$) of direction of attention (left versus right) and hemisphere of activation (left versus right) indicated that attention produced a contralateral fusiform gyrus activation.

FIG. 3 Correspondence between best-fit dipole of the P1 attention effect and locus of PET activation. Top, Graph represents the 'goodness-of-fit' between the dipole model and observed e.r.p. scalp distribution of attend-left minus attend-right difference waveform at different latencies (50–230 ms), plotted as scalp voltage variance not accounted for (that is, residual variance (RV)) by the seeded (upper trace) or best-fit inverse (lower trace) dipole models. For the seeded dipoles the location was fixed in the fusiform gyrus, but dipole orientations were permitted to vary. Fitting was done over 20-ms windows centred on the time points indicated. In the case of the best-fit inverse solutions, for each time epoch we fit a separate unconstrained best inverse solution for the dipole pair with starting positions in the fusiform gyrus. Lower values of residual variance indicate a close correspondence between the recorded e.r.p. attention effects and the model data. The seeded dipoles accounted for 96.2% of variance in the 110–130 ms interval. The best-fit dipole locations (red circles) were shifted slightly from the centre of the PET activation, but were still in the region of PET activation (black circle); these dipoles explained 98.4% of variance in the 110–130 ms interval. The interval of the best fit corresponded to the peak of the P1 attention effect. Outside this time period the residual variance rose substantially, and the loci of the best-fit inverse solutions (green circles) were variable in their distance from the fusiform gyrus activations. However, in the time range of the P1 attention effect the best-fit inverse dipoles converged on the locus of PET activation, as shown for right hemisphere dipole locations on a Talairach section²⁸ through the fusiform gyrus (bottom).

These 'seeded' dipoles provided a good account of the e.r.p. attention difference maps in the latency range of the P1 attention effect (compare Fig. 2b right, with Fig. 2c right). When the fixed-location constraint on the bilateral dipoles was released to permit the modelling algorithm to obtain the smallest residual difference between model and recorded e.r.p. data, a similarly close correspondence with the observed e.r.p. attention effects was obtained

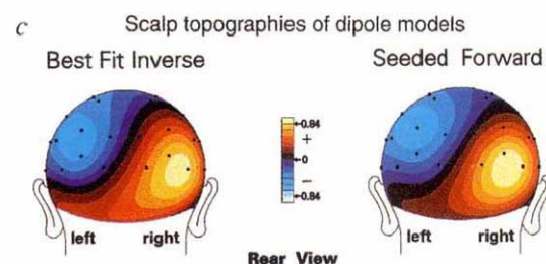
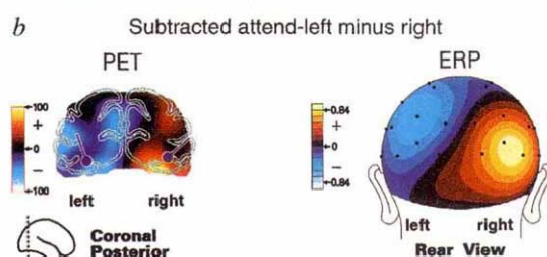
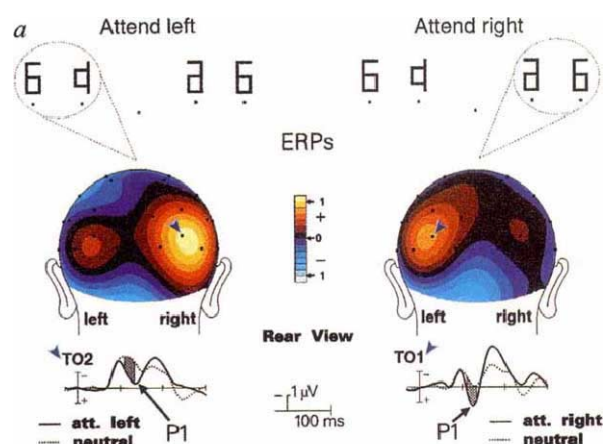
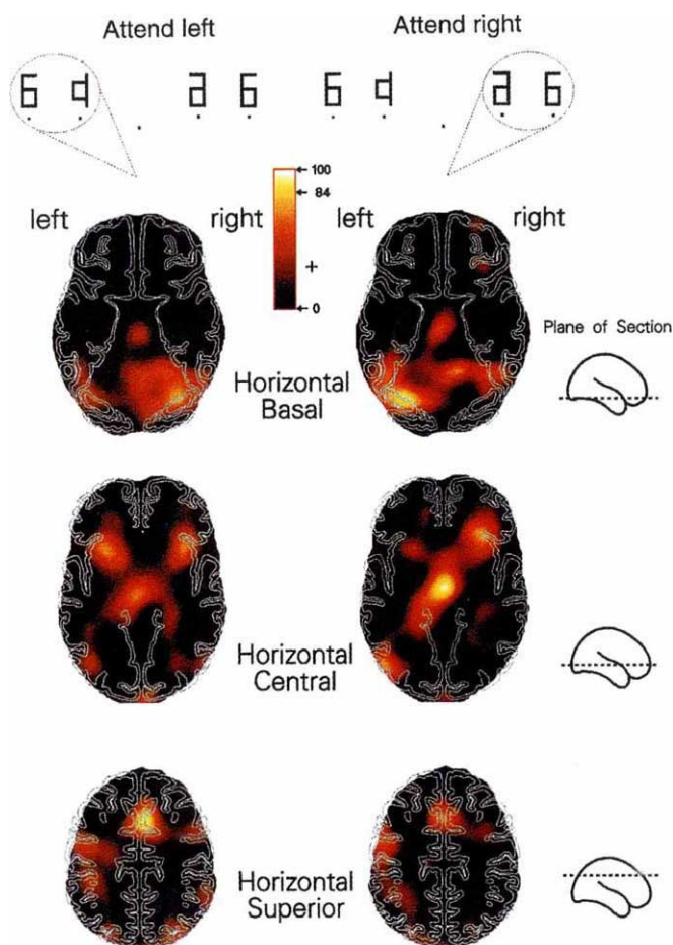


FIG. 2 Comparison of PET and e.r.p. attention effects. The stimuli and tasks were the same in separate PET and e.r.p. sessions, but in the e.r.p. session the subjects received 10 runs in each condition. **a**, Topographic isovoltage contour maps of the P1 attention effect for attend-left minus passive (left column) and attend-right minus passive (right column) conditions: scale is in microvolts. Below each topographic map are the corresponding e.r.p. waveforms and attention effects (ANOVA, 80–110 and 110–130 ms windows, both $P < 0.01$ for direction of attention by hemisphere interaction) recorded from contralateral occipital scalp sites indicated by the arrows (TO1 and TO2 are halfway from O1 to T5, and O2 to T6 of the International 10–20 System, respectively); shading indicates time period of the maps. **b**, At left is a coronal section through the posterior occipital region showing the attention-specific PET activation in the fusiform gyrus in the attend-left minus attend-right difference image. The fusiform gyrus activations in these subtraction images were significant for both the right fusiform gyrus activation ($t = 4.3$, $P < 0.001$) and the left fusiform gyrus activation ($t = -2.6$, $P < 0.02$); note that the left fusiform gyrus activation is seen as a relative decrease in the figure because of the direction of the subtraction. The dots represent the locations of the best inverse dipole solutions, with dipole orientations indicated by the attached lines. The corresponding attend-left minus attend-right topographic maps for the P1 attention effect are shown at right. **c**, At right, the topographic map derived from model dipolar sources seeded in the centre of the PET activation in fusiform gyrus over the 80–130 ms time range provided a close fit to the recorded P1 attention effect (b, right column). The constraint-free best-fit dipoles moved slightly anteriorly and laterally, and the topographic maps showed only minor alterations (left column). For detailed e.r.p. methodology, see ref. 11.

(compare Fig. 2*b* right, with Fig. 2*c* left). Thus, the localization of the model dipoles in the region of the PET activation in the posterior fusiform gyrus (Fig. 3) produced a model scalp field with a topography that was highly similar to that of the early P1 attention effect that was actually recorded.

The results from these combined PET and e.r.p. recordings converge in several key respects. First, in line with previous e.r.p. studies in humans^{7,14}, neither the e.r.p. nor PET measurements provided any evidence that spatial attention influences processing in the striate cortex or in the afferent geniculostriate pathways. Second, PET and e.r.p. recordings both showed that neural activity was selectively modulated in the ventral extrastriate visual cortex contralateral to the direction of attention, and that this modulation could be dissociated from nonspecific factors such as arousal or alertness. Finally, dipolar neural generators at the locus of the PET activity in the fusiform gyrus provided a good account of the early ERP attention effect at the scalp between 80–130 ms latency, suggesting that these PET and e.r.p. effects arose from a common source of neural activity.

The finding of attentional modulations in the fusiform gyrus at 80–130 ms latency indicates that spatial attention influences activity in the human occipito-temporal or 'ventral stream' of visual cortical processing to permit inputs from attended regions to gain preferential access to higher stages of feature analysis, pattern identification and object recognition^{15–17}. Similar attentional modulations of neural activity in ventral stream cortical areas have been observed in neurophysiological studies of non-human primates^{18–21}. These attentional mechanisms appear to be controlled by a network of brain structures that includes the posterior parietal lobe, anterior cingulate cortex, and the pulvinar nucleus of the thalamus^{3, 5, 22–26}. In a previous study that involved the repetitive shifting of attention between locations, activation of the parietal lobes was a prominent finding using PET²⁵. Presumably, such parietal activation was absent in our study because attention was sustained on a given location for several minutes rather than continually shifted between locations. Thus, it appears that sustained focal attention to location engages stimulus selection processes in the ventral extrastriate cortex with minimal activation of the parietal lobes.

Together, these PET and e.r.p. data provide a high-resolution view in both temporal and spatial domains of the earliest changes in visual processing that accompany visual-spatial selective attention in humans. The convergent use of different neuroimaging methods such as PET and e.r.p. recording promises to be a powerful approach for defining the spatial-temporal properties of brain activity underlying cognitive processes such as selective attention. □

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Nitric oxide in skeletal muscle

Lester Kobzik*, Michael B. Reid†, David S. Brett‡ & Jonathan S. Stamler§||

* Department of Pathology, Brigham and Women's Hospital and Physiology Program, Harvard School of Public Health, Boston, Massachusetts 02115, USA

† Pulmonary and Critical Care Medicine, Baylor College of Medicine, Houston, Texas 77030, USA

‡ Department of Physiology, UCSF, San Francisco, California 94143, USA

§ Department of Medicine, Pulmonary and Cardiovascular Divisions, and Department of Cell Biology, Duke University Medical Center, Box 3177, Durham, North-Carolina 27710, USA

REACTIVE oxygen intermediates modulate skeletal muscle contraction^{1,2}, but little is known about the role of nitric oxide (NO). Here we show that rat skeletal muscle expresses neuronal-type NO synthase and that activity varies among several respiratory and limb muscles. Immunohistochemistry showed prominent staining of type II (fast) fibre cell membranes with antibodies against neuronal-type NO synthase. NO synthase activity in muscles correlated with type II fibre density. Resting diaphragm muscle produced detectable NO_x, but no reactive oxygen intermediates. In contrast, actively contracting muscle generated increased levels of reactive oxygen intermediates. Contractile function was augmented by blockers of NO synthase, extracellular NO chelation, and guanylyl cyclase inhibition; it was depressed by NO donors and by increased levels of cyclic GMP. Force-frequency plots of different muscles showed an inverse correlation between NO synthase activity and force development. Our results support two physiological functions of NO in skeletal muscle. The first is to promote relaxation through the cGMP pathway^{3,4}. The second is to modulate increases in contraction that are dependent on reactive oxygen intermediates and which are thought to occur through reactions with regulatory thiols on the sarcoplasmic reticulum^{5,6}.

During analysis of neuronal-type NO synthase (nc-NOS) localization in rat trachea⁷, we observed prominent immunostaining of adjacent oesophageal skeletal muscle. Encouraged by the observation that nc-NOS messenger RNA is expressed in human skeletal muscle samples⁸, we investigated the production, source and function of NO in mammalian (rat) skeletal muscle.

Biochemical assays of skeletal muscle homogenates showed NO synthase activity (Table 1) was associated with the membrane fraction. Western staining with anti-nc-NOS identified an immunoreactive band of the predicted relative molecular mass (*M_r* 160 K, not shown) which comigrated with NO synthase from rat cerebellum.

The NO synthase activity of individual muscles correlated strongly with type II fibre composition^{9,10} (Table 1). Immunolocalization showed that nc-NOS antigens were prominent in

|| To whom correspondence should be addressed.

the sarcolemma of some, but not all, muscle fibres (Fig. 1a). Histochemistry identified NO-synthase-positive fibres as type II (fast) fibres (Fig. 1b), consistent with observations that NO-synthase activity increases as the proportion of type II (NO-synthase-positive) fibres increases (Table 1).

We measured contractile properties of muscles that span the ranges of fibre type and NO synthase activity shown in Table 1. In soleus, diaphragm and extensor digitorum longus muscles, the position of the force-frequency relationship was shifted to the right as NO-synthase activity increased, suggesting that NO may inhibit force development (Fig. 2a).

We used three inhibitors of NO synthase (7-nitroindazole, aminoguanidine, and nitro-L-arginine) to assess the role of endogenous NO on contractile function in diaphragm muscle. Each inhibitor caused a significant shift to the left of the force-frequency relationship (Fig. 2b). However L-arginine at similar concentrations (up to 1 mM) had no effect on contraction. Higher

concentrations of L-arginine inhibited force development (in the presence of NO-synthase inhibitors) but persistent inhibition after drug washout and other nonspecific effects limit this interpretation. Contractile increases caused by NO-synthase inhibition were reversed by the exogenous NO donors *S*-nitroso-*N*-acetylcysteine (SNAC) and sodium nitroprusside (100 μ M, Fig. 2c); inhibitory effects were also seen with *S*-nitroso-glutathione and diethylamine-NO (data not shown). In addition, haemoglobin, which traps NO, enhanced relative force production at 40 Hz (50 μ M haemoglobin, $n=5$, $P=0.062$; 50–250 μ M haemoglobin, $n=10$, $P<0.001$).

As NO may act via the cGMP signalling pathway^{3,4}, we investigated agents that increase intracellular cGMP, including 8-bromo-cGMP (8-Br-GMP), a cGMP analogue, and dipyrindamole, a phosphodiesterase inhibitor. Both agents reversed the contractile increases caused by inhibition of NO synthase to a small (but significant) degree (Fig. 2d). Greater effects were seen with a combination of 8-Br-GMP and dipyrindamole (Fig. 2d). This is noteworthy because dipyrindamole can also increase cAMP levels, which in turn increases the extent of contraction of skeletal muscle^{11–13}. The guanylyl cyclase inhibitor LY83583 (5–10 μ M) increased relative force production during submaximal tetanic contraction ($P<0.001$; data not shown). Similar increases with methylene blue are interpreted with caution in the light of its potential toxicity over time. Measurements of cGMP by enzyme-linked immunosorbent assay (ELISA) showed significant increases above control by the addition of SNAC (1 mM), (pmol mg^{-1} : control, 0.27 ± 0.01 ; SNAC, 0.55 ± 0.05 , $N=6$, $P<0.001$). Comparable results were seen with nitroprusside, consistent with earlier reports¹⁴. Immunohistochemistry confirmed the presence of cGMP in the immediate vicinity of NO synthase (Fig. 1c).

As we found relatively modest effects of cGMP, and previous work has shown that reactive oxygen intermediates (ROIs) regulate skeletal muscle function^{2,6,15}, we examined production of NO and superoxide (O_2^-) by diaphragm bundles. We employed the cytochrome *c* assay which has been used to detect O_2^- (in skeletal muscle¹) and NO (in bacteria^{16–18}). Resting diaphragm bundles released reducing equivalents (mean rate of cytochrome *c* reduction, 2.8 ± 0.6 pmol per mg muscle per min

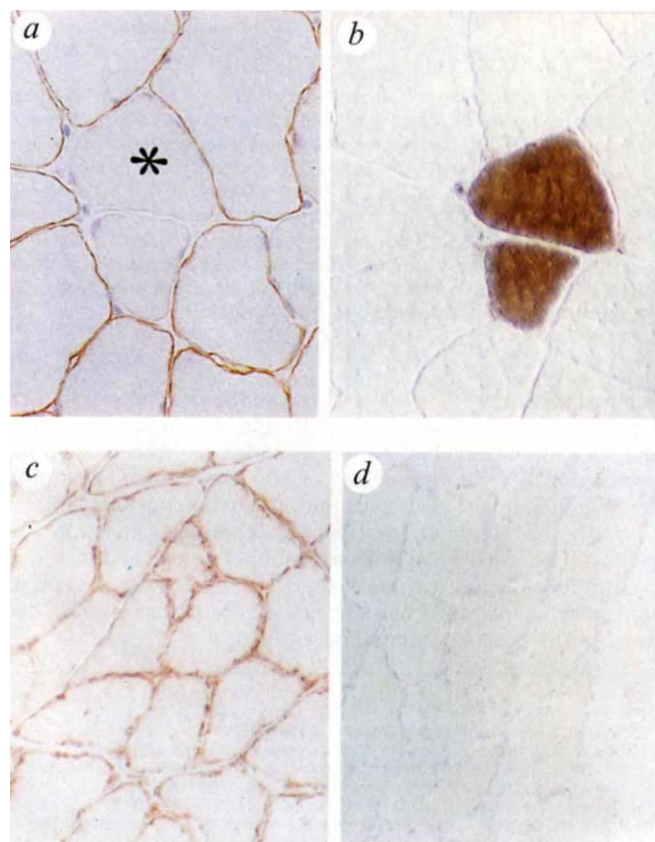


FIG. 1 Localization of nitric oxide synthase (NOS) antigen and cGMP in skeletal muscle. a, NOS immunostaining. Immunohistochemical labelling, using polyclonal anti-rat nc-NOS²⁷ of cryostat sections of rat skeletal muscle, showed distinct labelling of muscle fibre surface membranes in many but not all fibres (asterisk marks negative fibres). Controls including irrelevant primary antibody or buffer showed no labelling (not shown). Similar results were obtained using a separate nc-NOS-specific antibody, kindly provided by T. Dawson. b, Muscle histochemistry. Staining of adjacent serial sections for ATPase identified NOS-positive fibres as type II (fast), and NOS-negative fibres as type I (slow). c, cGMP immunostaining. Prominent staining of cGMP is seen in the area of the sarcolemma, supporting the possibility that NOS and guanylate cyclase activities may be coupled. d, cGMP control. No staining is observed with control mouse IgG.

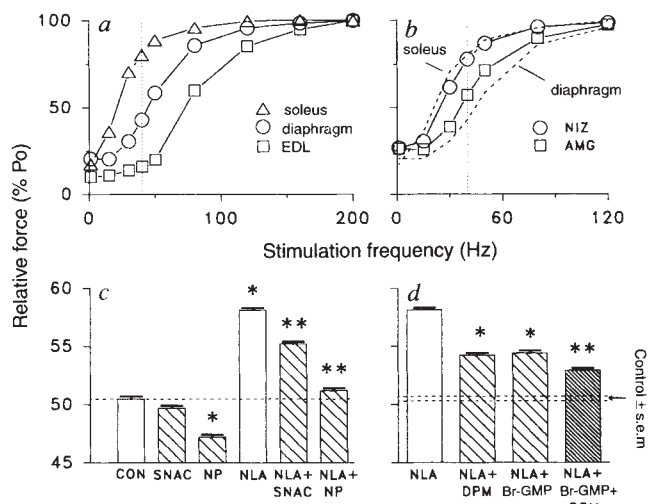
METHODS. Immunohistochemical labelling was done as previously described, using an avidin-biotin peroxidase complex method and a specific rabbit anti-rat brain NOS antibody^{7,27}, or using a cGMP-specific monoclonal antibody²⁸, provided by M. Kaliner. For ATPase typing, a standard histochemical protocol was followed, including preincubation of sections at pH 9.4, 4.6 and 4.2 to optimize differentiation of fibre types. The results shown are from a sample preincubated at pH 4.2 which gives intense labelling of type I fibres only.

TABLE 1 Skeletal muscle NO synthase activity correlates with fibre composition

Tissue	NO synthase activity (pmol min^{-1} mg^{-1} protein)		Muscle fibre composition (% type II)
	Experiment 1	Experiment 2	
Cerebellum	145.0	158.0	—
Extensor digitorum longus	13.5	13.7	98 $\pm$ 7
Gastrocnemius	11.0	12.0	93 $\pm$ 5
Plantaris	14.0	9.5	91 $\pm$ 3
Diaphragm	5.2	5.0	60 $\pm$ 1
Soleus	2.5	2.5	13 $\pm$ 4
Heart	0.9	1.3	—

NO synthase activity was identified in a panel of rat muscles by monitoring the conversion of ^3H -arginine to ^3H -citrulline. Published values for type II composition^{9,10} are shown and correlate strongly with average NO synthase activity ($r=0.97$; $P<0.001$). Rats were killed and the lower limb muscles, diaphragm, heart and cerebellum were dissected out. Tissues were homogenized in 40 volumes of homogenization buffer containing 25 mM Tris-HCl (pH 7.4) and 1 mM EDTA. The homogenate was centrifuged at 4 $^{\circ}\text{C}$ for 15 min at 20,000g. The pellet was resuspended in half the original volume of homogenization buffer. 25 μ l of homogenate was added to 25 μ l of 100 nM ^3H -arginine (50 Ci mmol^{-1}) and 100 μ l of assay buffer containing 50 mM HEPES (pH 7.4), 1 mM NADPH, 1 mM EDTA and 1.25 mM CaCl_2 . After incubation for 15 min at 22 $^{\circ}\text{C}$, assays were terminated with 2 ml of 20 mM HEPES (pH 5.5), 2 mM EDTA. Samples were applied to 1 ml columns of Dowex AG50WX-8 (Na^+ -form) which were eluted with 2 ml of water. ^3H -citrulline was quantified by liquid scintillation spectroscopy of the 4 ml flow-through. Results of two separate experiments are shown.

FIG. 2 NO depresses contractile function of skeletal muscle. **a**, Contractile properties of muscles. Data from soleus ($n=6$), diaphragm ($n=7$), and extensor digitorum longus (EDL; $n=5$) show that mean forces ($\pm$ s.e.m.) developed during submaximal contractions (stimulus frequencies of 15–150 Hz) decrease as NOS activity increases (soleus < diaphragm < EDL; Table 1). A vertical dotted line identifies the frequency (40 Hz) at which force was recorded for regression calculations (see text). Maximal tetanic stresses (P_0 ; stimulated at 200 Hz) in $N\text{ cm}^{-2}$ were 29.9 ± 2.2 for soleus, 24.2 ± 1.0 for diaphragm, 30.9 ± 2.6 for EDL. **b**, Contractile effects of NOS inhibitors. Exposure to the NOS inhibitors 7-nitroindazole (NIZ) 1 mM ($n=3$ per frequency) or amino-guanidine (AMG) 500 μM ($n=3$ per frequency) shifted the diaphragm force–frequency relationship (lower dashed curve) up and to the left ($P<0.0001$; ANOVA). In the case of NIZ, the curve is indistinguishable from the relationship measured in native soleus, a slow-twitch postural muscle (upper dashed curve). P_0 , 23.4 ± 0.3 and $25.0 \pm 0.6\text{ N cm}^{-2}$ (means $\pm$ s.e.m.) for NIZ and AMG, respectively, which are not different from diaphragm. Significant shifts of a lesser magnitude were produced by NIZ 100 μM , AMG 200 μM and NLA 1 mM ($P<0.05$ for all; data not shown). The vertical dotted line depicts the frequency used in **c** and **d** to test pharmacological effects. **c**, Inhibitory effects of NO donors. Relative forces ($\pm$ s.e.m.) developed at 40 Hz are shown for diaphragm fibre bundles ($n=5$ –6 per group) treated with S-nitroso-*N*-acetylcysteine (SNAC), 100 μM , sodium nitroprusside (NP) 100 μM , nitro-L-arginine (NLA) 10 mM, NLA plus SNAC, or NLA plus NP. Dashed line depicts the mean force developed in native muscle control (CON). P_0 ($N\text{ cm}^{-2}$): 26.3 ± 0.6 , CON; 27.2 ± 0.8 , SNAC; 26.9 ± 0.3 , NP; 27.0 ± 0.6 , NLA; 28.1 ± 0.7 , NLA + SNAC; 27.6 ± 0.1 , NLA + NP. * Different from control ($P<0.0001$); ** different from NLA ($P<0.0001$). SNAC and NLA + NP were not different from control. **d**, Inhibitory effects of cGMP after NOS blockade. Relative forces ($\pm$ s.e.m.) developed by diaphragm fibre bundles stimulated at 40 Hz ($n=5$ –6 per group) are shown for native muscle (dashed lines) and muscle treated with NLA (transcribed from **c**), NLA



plus dipyrindamole (DPM) 10 μM , NLA plus 8-Br-GMP 1 mM, or NLA plus DPM and 8-Br-GMP. NLA plus LY83583 increased force above that with NLA alone ($P<0.001$; not shown). All values were greater than control ($P<0.0001$). P_0 ($N\text{ cm}^{-2}$): 26.3 ± 0.6 , control; 27.0 ± 0.6 , NLA; 27.6 ± 0.5 , NLA + DPM; 27.0 ± 1.1 , NLA + 8-Br-GMP; 27.0 ± 0.4 , NLA + 8-Br-GMP + DPM. * Different from NLA and from NLA + BGM + DPM ($P<0.0001$); ** different from all other groups ($P<0.0001$). Isolation and stimulation of muscle are described elsewhere². In **a** and **b**, protocol 2, ref. 2 was used. In **c** and **d**, 40 Hz and 200 Hz forces were measured every 15 min for 1 h, following drug equilibration.

at 37 °C, $n=25$, time 60–90 min). Generation of reducing equivalents was lowered by inhibition of NO-synthase with nitro-L-arginine (1 mM) ($67 \pm 18\%$ of control; $P<0.05$), an effect abolished by the addition of NO donors to muscle treated with nitro-L-arginine (data not shown). In contrast, superoxide dismutase (SOD) (10^3 U ml^{-1}) and catalase (10^3 U ml^{-1}) did not affect basal production of reducing equivalents ($106 \pm 82\%$ control). This contrasts sharply with results obtained from actively contracting muscle (6 contractions per min at 25 Hz for 1 h) in which cytochrome *c* reduction is increased more than sixfold relative to passive muscle (active muscle, $672 \pm 83\%$ control; $P<0.001$), 85% of this increase being attributable to SOD-sensitive ROIs (active muscle + SOD, $188 \pm 41\%$ control; $P<0.001$ compared with active muscle²). Reduction of cytochrome *c* may result from the direct effect of NO_x^{16-18} or indirectly through effects on other systems, such as enzymes that influence redox state¹⁹.

In summary, we have found that: skeletal muscle contains a neuronal-type NOS restricted to the sarcolemma of type II fibres; NO acts to inhibit force production; these effects are mediated, at least in part, through increases in cGMP; and that established mechanisms of redox regulation of muscle are likely also to involve NO_x .

We propose a model in which NO influences skeletal muscle contraction by two related mechanisms. The first is through the cGMP signalling pathway that was not previously known to inhibit skeletal muscle function. This pathway may be active in resting muscle, and perhaps augmented during contraction by Ca^{2+} stimulation of nc-NOS. The second is by interactions with endogenous ROIs which (reversibly) enhance contractile function in unfatigued muscle². Reactive oxidants are known to modulate Ca^{2+} flux at the sarcoplasmic reticulum by redox-sensitive thiol targets on Ca^{2+} release channels^{5,6} and the Ca^{2+} -dependent ATPase^{20,21}. This resembles the mechanism of regulation of ligand-gated Ca^{2+} flux recently described for NO_x in other systems²². In the actively contracting state, increased production of ROIs will probably divert NO away from metal centres (for example, guanylyl cyclase, which inhibits contraction) towards

sulphydryl targets²³⁻²⁶ (such as those on the sarcoplasmic reticulum) through formation of NO_x . Resultant S-nitrosylation and thiol oxidation reactions²⁴ would promote Ca^{2+} release and force production^{5,6,20,21}. Other effects of NO, on cellular respiration for example, might also contribute to changes in muscle function. The inverse correlation between contractile function and NO-synthase activity may have pathophysiological and therapeutic consequences in view of changes in fibre types that occur in neuromuscular and connective tissue disorders as well as diseases of the heart and diaphragm. □

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Drug excretion mediated by a new prototype of polyspecific transporter

Dirk Gründemann, Valentin Gorboulev,
Stepan Gambaryan, Maike Veyhl
& Hermann Koepsell*

Anatomisches Institut, Bayerische Julius-Maximilians-Universität,
Koellikerstrasse 6, 97070 Würzburg, Germany

CATIONIC drugs of different types and structures (antihistaminics, antiarrhythmics, sedatives, opiates, cytostatics and antibiotics, for example) are excreted in mammals by epithelial cells of the renal proximal tubules and by hepatocytes in the liver¹⁻⁴. In the proximal tubules, two functionally disparate transport systems are involved which are localized in the basolateral and luminal plasma membrane and are different from the previously identified neuronal monoamine transporters and ATP-dependent multidrug exporting proteins^{1-3,5-12}. Here we report the isolation of a complementary DNA from rat kidney that encodes a 556-amino-acid membrane protein, OCT1, which has the functional characteristics of organic cation uptake over the basolateral membrane of renal proximal tubules and of organic cation uptake into hepatocytes. OCT1 is not homologous to any other known protein and is found in kidney, liver and intestine. As OCT1 translocates hydrophobic and hydrophilic organic cations of different structures, it is considered to be a new prototype of polyspecific transporters that are important for drug elimination.

Using functional expression cloning in *Xenopus* oocytes, we isolated a 1,882-base-pair (bp) cDNA from a rat kidney library, which induces high activity of N¹-methylthioacetamide (NMN)-inhibitable ¹⁴C-tetraethylammonium (¹⁴C-TEA) uptake (Fig. 1a). This clone, OCT1, contains an open reading frame encoding

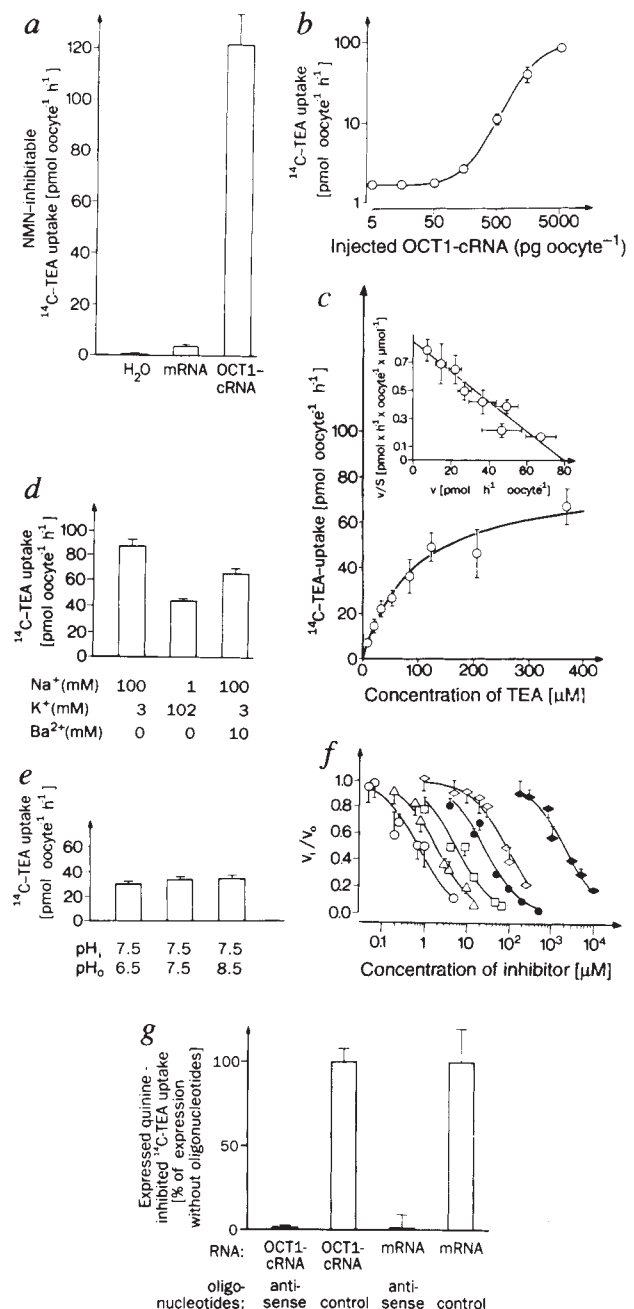


FIG. 1 Expression of OCT1 in *Xenopus* oocytes. Oocytes were injected with 3 ng (c, d, e), 5 ng (f, g) or 10 ng OCT1-cRNA (a). The indicated ¹⁴C-TEA-uptake rates represent the medians of 10–20 oocytes $\pm$ s.e.m. a, Comparison of NMN-inhibitable ¹⁴C-TEA uptake after injection of water, 20 ng rat kidney mRNA or 10 ng OCT1-cRNA; ¹⁴C-TEA and NMN concentrations in incubation medium were 200 μ M and 10 mM respectively. Under these conditions 77 $\pm$ 4% of the total uptake was inhibited by NMN. b, Uptake rates of 200 μ M ¹⁴C-TEA after injection of different amounts of OCT1 cRNA. The curve was calculated by fitting the Hill equation to the data ($n = 1.9 \pm 0.2$). c, Substrate dependence of ¹⁴C-TEA uptake expressed by OCT1-cRNA. Uptake measured with cRNA-injected oocytes was corrected for that obtained with water-injected control oocytes, which increased linearly with substrate concentration (30 fmol h⁻¹ oocyte⁻¹ μ M⁻¹). The curve was fitted to the Michaelis-Menten equation ($K_m = 95 \pm 10$ μ M; $V_{max} = 81 \pm 5$ pmol h⁻¹ oocyte⁻¹). d, Dependence of expressed ¹⁴C-TEA uptake on membrane potential. In oocytes injected with OCT1 cRNA, the uptake of 95 μ M ¹⁴C-TEA was measured in the presence of Na⁺, K⁺ and Ba²⁺ at the indicated concentrations and corrected for uptake in water-injected control oocytes. Membrane potentials measured by conventional two-microelectrode techniques ranged between -40 and -60 mV (100 mM Na⁺, 3 mM K⁺), 0 and -10 mV (1 mM Na⁺, 102 mM K⁺), and -18 and -22 mV (100 mM Na⁺, 3 mM K⁺, 10 mM Ba²⁺). e, Uptake of 95 μ M ¹⁴C-TEA in the absence and presence of proton gradients in OCT1-cRNA-injected oocytes. To prevent proton-gradient-induced changes in membrane potential, measurements were made in the presence of 102 mM K⁺ and 1 mM Na⁺. Measurements with an ion-sensitive microelectrode showed that the internal pH changed by less than 0.1 units during the 30-min uptake recording. f, Inhibition of OCT1-mediated uptake of 95 μ M ¹⁴C-TEA by decynium-22 (○), quinine (△), desipramine (□), procainamide (●), O-methylisoprenaline (◇) and tetramethylammonium

(◆). g, Inhibition of quinine-inhibitable ¹⁴C-TEA uptake, expressed by mRNA from rat kidney, by an antisense oligonucleotide directed against OCT1. Poly(A)⁺ mRNA and OCT1 cRNA were incubated without and with 40 μ M antisense oligonucleotide (5'-AGG ACA TCA TCC ACG GTG GG-3', nucleotides 60–40 of OCT1) or with a non-interacting control oligonucleotide (control) as described¹⁷. 50 ng mRNA per oocyte was injected, the uptake of 200 μ M ¹⁴C-TEA measured in the absence and presence of 25 μ M quinine, and the quinine-inhibited uptake calculated.

METHODS. *Xenopus laevis* oocytes were selected and expression measured as described²⁴. After RNA injection, oocytes were incubated for 3 days with 5 mM Tris-HCl, pH 7.8, 100 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂ (ORI buffer). Uptake was measured for 90 min at 22 °C by incubating the oocytes with ¹⁴C-TEA in ORI buffer, ORI with altered Na⁺ and K⁺, ORI plus Ba²⁺, ORI with altered pH (HEPES substituted for Tris), or ORI plus inhibitors. OCT1-expressed TEA uptake was linear for more than 90 min. For measurements with altered concentrations of Na⁺, K⁺, H⁺ and with inhibitors, oocytes were pre-incubated for 30 min under the respective buffer conditions and the uptake rate was then determined during a 30-min incubation with ¹⁴C-TEA.

* To whom correspondence should be addressed.

a membrane protein of relative molecular mass 61,528, which shares no similarity with any protein in the data banks (Fig. 2a). Expression of ^{14}C -TEA uptake in oocytes was dependent on the amount of injected OCT1 cRNA (Fig. 1b), and the substrate dependence of OCT1-mediated ^{14}C -TEA uptake followed Michaelis-Menten kinetics (Fig. 1c). The estimated K_m value of $95 \pm 10 \mu\text{M}$ was similar to the K_m (160 μM) for cation transport through the basolateral membrane of rat renal proximal

tubules¹³ and was more than ten times lower than the apparent K_m of the H^+ -cation antiport through the luminal membrane^{2,14}. To determine whether OCT1 could be responsible for potential-dependent organic cation transport over the basolateral membrane or the luminal H^+ -organic cation antiport^{1,3,8,11}, we investigated whether OCT1-mediated uptake is potential- or proton-gradient-dependent and studied the inhibition of ^{14}C -TEA uptake using a variety of inhibitors. Figure 1d and e shows that

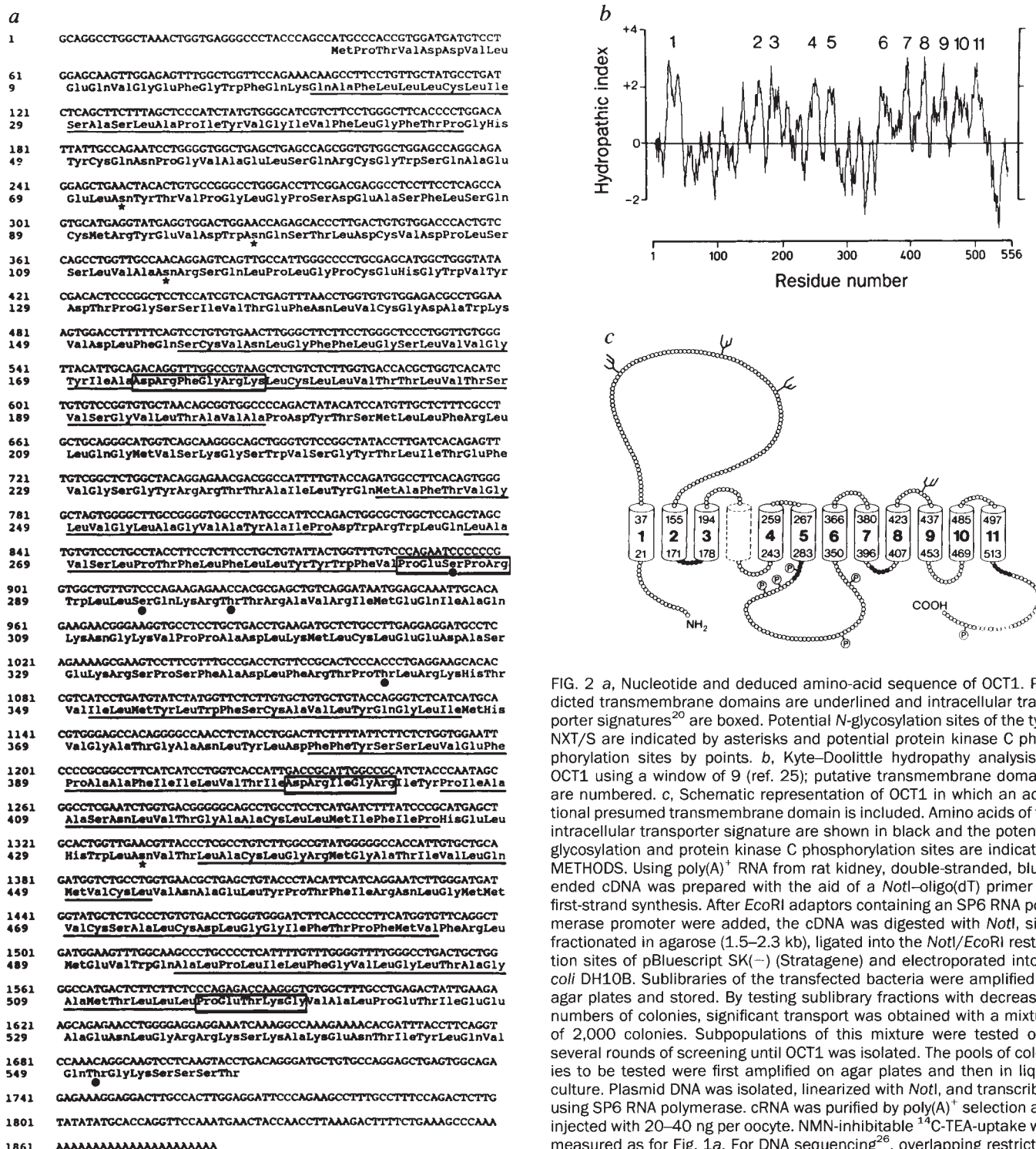


FIG. 2 a, Nucleotide and deduced amino-acid sequence of OCT1. Predicted transmembrane domains are underlined and intracellular transporter signatures²⁰ are boxed. Potential N-glycosylation sites of the type NXT/S are indicated by asterisks and potential protein kinase C phosphorylation sites by points. b, Kyte-Doolittle hydropathic analysis of OCT1 using a window of 9 (ref. 25); putative transmembrane domains are numbered. c, Schematic representation of OCT1 in which an additional presumed transmembrane domain is included. Amino acids of the intracellular transporter signature are shown in black and the potential glycosylation and protein kinase C phosphorylation sites are indicated. METHODS. Using poly(A)⁺ RNA from rat kidney, double-stranded, blunt-ended cDNA was prepared with the aid of a NotI-oligo(dT) primer for first-strand synthesis. After EcoRI adaptors containing an SP6 RNA polymerase promoter were added, the cDNA was digested with NotI, size-fractionated in agarose (1.5–2.3 kb), ligated into the NotI/EcoRI restriction sites of pBluescript SK(–) (Stratagene) and electroporated into *E. coli* DH10B. Sublibraries of the transfected bacteria were amplified on agar plates and stored. By testing sublibrary fractions with decreasing numbers of colonies, significant transport was obtained with a mixture of 2,000 colonies. Subpopulations of this mixture were tested over several rounds of screening until OCT1 was isolated. The pools of colonies to be tested were first amplified on agar plates and then in liquid culture. Plasmid DNA was isolated, linearized with NotI, and transcribed using SP6 RNA polymerase. cRNA was purified by poly(A)⁺ selection and injected with 20–40 ng per oocyte. NMN-inhibitable ^{14}C -TEA-uptake was measured as for Fig. 1a. For DNA sequencing²⁶, overlapping restriction fragments of OCT1 were re-cloned and completely sequenced on both strands using universal and specific primers with Sequenase version 2.0 kit (US Biochem.).

the ^{14}C -TEA uptake expressed by OCT1 is potential-dependent but is not significantly altered by inwardly or outwardly directed proton gradients of 1 pH unit. Thus OCT1 has virtually the same transport characteristics as organic cation transport measured over the basolateral membrane of renal proximal tubules. Figure 1f and Table 1 show that OCT1-mediated ^{14}C -TEA uptake is inhibited by cations having different molecular structures, including several common drugs. Unlike the multidrug transporter, which is inhibited exclusively by hydrophobic substances⁷, OCT1 is also inhibited by hydrophilic compounds like tetramethylammonium (TMA) and NMN. We found that desipramine inhibited OCT1 700-fold less effectively than the neuronal plasma-membrane noradrenaline transporter¹⁵ and 5 μM of reserpine did not alter OCT1-mediated transport, whereas the vesicular monoamine transporters are inhibited by subnanomolar reserpine concentrations⁵. Comparing the low-affinity inhibitors TMA and NMN, we found that the K_i values of OCT1-expressed transport ($\sim 1\text{ mM}$) were identical to the K_i values (TMA, $1.4 \pm 0.4\text{ mM}$; NMN, $1.1 \pm 0.2\text{ mM}$) estimated for the basolateral TEA uptake in rat renal proximal tubules¹³, but differed significantly from the K_i values (TMA, $74 \pm 48\text{ mM}$; NMN, $8.3 \pm 2.7\text{ mM}$) estimated for luminal TEA uptake (ref. 14 and K. J. Ullrich *et al.*, unpublished results). The connection of OCT1 with basolateral transport is also supported by the K_i of 0.4 μM for inhibition of OCT1-mediated uptake by decynium-22: in LLC-PK1 kidney cells, K_i for the inhibition of TEA transport was 6 nM apically and $>0.1\text{ }\mu\text{M}$ basolaterally¹⁶. To verify that structurally different organic cations can be transported by OCT1, uptake of *N*-methyl-4-phenylpyridinium (MPP) was measured. After injection of 8 ng OCT1 cRNA per oocyte in the same batch of oocytes, similar V_{max} values were estimated for the expressed, quinine-inhibitable uptake of ^{14}C -TEA ($148 \pm 4\text{ pmol oocyte}^{-1}\text{ h}^{-1}$) and ^3H -MPP ($97 \pm 5\text{ pmol oocyte}^{-1}\text{ h}^{-1}$). We obtained $\sim 100\%$ inhibition by antisense oligonucleotides to OCT1¹⁷ of the quinine-inhibitable uptake of 200 μM

^{14}C -TEA expressed by rat kidney mRNA (Fig. 1g). As luminal transport activity is less than 10% in the transport assay, our results suggest that OCT1 mediates organic cation transport across the basolateral membrane.

The nucleotide and derived amino-acid sequence of OCT1 is shown in Fig. 2a. The open reading frame is preceded by stop codons and a Kozak-type¹⁸ initiation site of translation (CAGCCATG). Hydropathy analysis of OCT1 (Fig. 2b) suggests that there are eleven putative transmembrane protein regions (TM). Because three *N*-linked glycosylation sites are predicted in the first hydrophilic loop, the amino terminus may lie in the cytoplasm. Another putative transmembrane domain

FIG. 3 Localization of OCT1-homologous mRNA analysed by northern blotting (a–d) and *in situ* hybridization (e–j). Northern blot hybridizations with the cDNA encoding OCT1 are shown in a and b, and control hybridizations with a cDNA encoding glyceraldehyde-3-phosphate dehydrogenase in c and d. For *in situ* hybridization, rat kidney cortex (e, f), liver (g, h) and small intestine (i, j) were hybridized with antisense (e, g, i) and sense probes of OCT1 cRNA (f, h, j). Specific signals were detected in renal proximal tubules, hepatocytes and small intestinal enterocytes. In kidney (e) no specific signals were detected in glomeruli (G), distal tubules (arrowhead) and collecting ducts (arrow). Scale bars, 100 μm . METHODS. Total RNA was isolated by the acid guanidinium-phenol-chloroform method²⁷ and mRNA purified using oligo(dT)-cellulose chromatography. For northern blotting, mRNA (tissues and 293 cells (5 μg), Caki-1 cells and LLC-PK1 cells (1.5 μg)) was fractionated on a formaldehyde-agarose gel, transferred to Hybond-N membrane (Amersham) and hybridized to a random-primed ^{32}P -labelled cDNA fragment of pOCT1 (nucleotides 285–1,196) for 18 h at 42 $^{\circ}\text{C}$ in 50% formamide, 5 $\times$ SSPE, 5 $\times$ Denhardt's solution, 0.5% SDS and 20 $\mu\text{g ml}^{-1}$ salmon sperm DNA. The blot was washed to a final stringency of 0.25 $\times$ SSPE, 0.1% SDS at 60 $^{\circ}\text{C}$. The lanes with mRNA from cells were exposed for 24 h and the other lanes for 6 h. The position of RNA standards (0.14–9.5 kb ladder; GIBCO/BRL) is indicated. To test the loading of mRNA analysed, the ^{32}P -OCT1 probe was removed and the filters were hybridized with ^{32}P -labelled human glyceraldehyde-3-phosphate dehydrogenase cDNA probe (ITC Biotechnology, Heidelberg). *In situ* hybridization was done on 7- μm cryostat sections of tissue fixed with 4% paraformaldehyde using digoxigenin-labelled sense and antisense cRNA probes. The probes were derived from nucleotides 396–835 and 396–1,205 of OCT1, respectively. For hybridization, sections were incubated for 12 h with the labelled cRNA probes ($3\text{--}5\text{ }\mu\text{g ml}^{-1}$) dissolved in 1 $\times$ Denhardt's solution containing 50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.3 M NaCl, 50% deionized formamide, 10% dextran sulphate and 0.5 mg ml^{-1} yeast tRNA. Sections were washed to a final stringency of 0.5 $\times$ SSC, 50% formamide (50 $^{\circ}\text{C}$), incubated (for 4 h at 22 $^{\circ}\text{C}$) with alkaline-phosphatase-labelled anti-digoxigenin antibodies, and developed with alkaline phosphatase substrate.

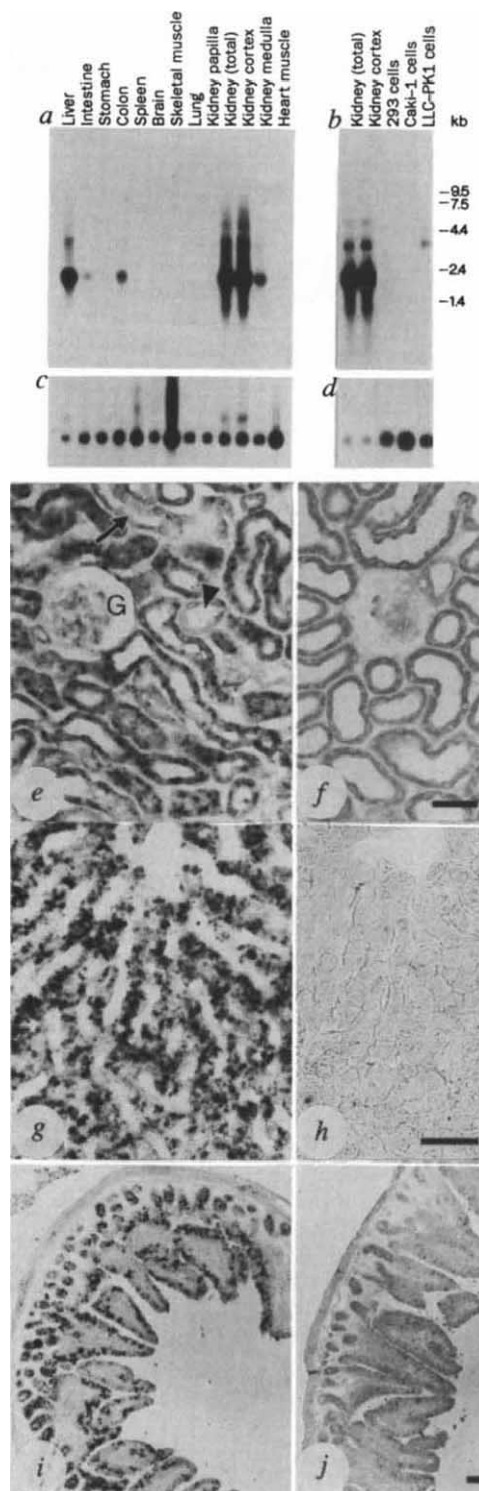


TABLE 1 Inhibitor sensitivity of ^{14}C -TEA uptake in *Xenopus* oocytes injected with cRNA of the renal organic cation transporter OCT1

Inhibitor	K_i (μM)
Cyanine-863	0.13 ± 0.02
Decynium-22	0.36 ± 0.08
Tetrapentylammonium	0.43 ± 0.09
Quinine	0.93 ± 0.08
Desipramine	2.8 ± 0.6
Mepiperphenidol	5.2 ± 0.3
Procainamide	13 ± 2
1-Methyl-4-phenylpyridinium	13 ± 2
Corticosteron	>10
Reserpine	>20
O-methyl-isoprenaline	43 ± 5
Tetramethylammonium	$1,000 \pm 100$
N ¹ -methylnicotinamide	$1,000 \pm 200$

Xenopus oocytes were injected with 5 ng OCT1 cRNA and the effect of 5–8 different inhibitor concentrations on the uptake of $95 \mu\text{M}$ ^{14}C -TEA into the oocytes was measured as for Fig. 1f. Inhibition curves were fitted by nonlinear regression analysis and the K_i values ($\pm\text{s.e.m.}$) calculated.

between TM3 and TM4 has been found in an OCT1-homologous cDNA from human kidney (our unpublished results) which may also exist in OCT1 (Fig. 2c). With this transmembrane domain included, there are several possible intracellular protein kinase C phosphorylation sites which may be involved in transport regulation¹⁹. Also, three short sequence motifs in OCT1 that have been identified in the cytoplasmic domains of transport proteins from different families²⁰ become localized intracellularly. Northern blot analysis (Fig. 3a–d) showed bands of 1.9, 3.4 and 4.8 kilobases (kb) in samples of kidney cortex, kidney medulla, liver, intestine and colon of rat. Porcine LLC-PK1 cells gave a single hybridization band at 3.4 kb. Extra-neuronal nor-adrenaline transport in heart and Caki-1 cells has been described that has the functional properties of renal luminal H^+ -cation antiporter^{21, 23}, but as we detected no hybridization in heart and Caki-1 cells, OCT1 probably belongs to a different genetic family. We amplified a cDNA fragment from rat liver by polymerase chain reaction which was identical to nucleotides 400–620 of OCT1, indicating that OCT1 is probably also expressed in liver. The uptake of organic cations in primary cultured rat liver hepatocytes has been found to be almost exclusively performed by a system with K_i values nearly identical to OCT1 for the inhibitors O-methyl-isoprenaline, MPP, quinine, decynium-22 and cyanine-863 (E. Martel, H. Russ, I. Azevedo and E. Schömig, manuscript in preparation). This OCT1 fingerprint of inhibitory constants indicates that OCT1 could be the main transporter responsible for organic cation uptake into hepatocytes. *In situ* hybridization in rat kidney showed that OCT1 is expressed in proximal tubules but apparently not in distal tubules, collecting ducts and glomeruli (Fig. 3e, f). OCT1 is expressed in hepatocytes in liver (Fig. 3g, h) and in enterocytes of villi and crypts in small intestine (Fig. 3i, j).

Our results indicate that OCT1 is a new type of polyspecific transporter which is involved in the elimination of cationic drugs. It shares common features with organic cation uptake over the basolateral membrane of renal proximal tubules and appears to be identical to the main organic cation uptake system in hepatocytes. Expression of the human OCT1-homologous gene and of the renal luminal organic cation transporter in epithelial cell lines will provide *in vitro* test systems for the development of drugs with optimized excretion and minimized nephrotoxicity. □

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Systemic and mucosal immunity induced by BCG vector expressing outer-surface protein A of *Borrelia burgdorferi*

Solomon Langermann*, Susan Palaszynski*, Ariadna Sadziene†, C. Kendall Stover*† & Scott Koenig*

* MedImmune Inc., Gaithersburg, Maryland 20878, USA

† Department of Microbiology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284, USA

THE bacillus Calmette–Guerin (BCG) is a live attenuated strain of *Mycobacterium bovis* which offers potential advantages as a vector for mucosal delivery of antigens^{1–3}. Recombinant BCG elicits protective humoral immune responses to a variety of antigens⁴. Furthermore, BCG binds specifically to microfold cells⁵ present in the epithelium overlying lymphoid follicles throughout the mucosal immune system^{6–8}. Here we show that a single intranasal vaccination with recombinant BCG expressing the outer-surface protein A antigen from *B. burgdorferi*⁹ results in a prolonged (more than one year) protective systemic IgG response and a highly sustained secretory IgA response which is disseminated throughout the mucosal immune system. Furthermore, intranasal immunization induces marked, organized lymphocyte accumulation in the proximal nasopharyngeal lymphoid tissue as well as at distal mucosal sites; the appearance and persistence of lymphoid aggregates correlates with the secretory immune responses. Thus

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† Present address: Pathogenesis Corporation, Seattle, Washington 98119, USA.

intranasal immunization with recombinant BCG is a powerful method for inducing long-lasting secretory and systemic immune responses.

We previously demonstrated that parenteral immunization of mice with an optimal dose of 2×10^6 CFU recombinant BCG expressing outer-surface protein A (rBCG-OspA) resulted in high levels of protective anti-OspA IgG antibodies¹⁰. These findings were consistent with earlier passive transfer studies showing that monoclonal and polyclonal sera specific for OspA protect mice against challenge with *B. burgdorferi*^{11,12}. Intranasal (i.n.) delivery of 2×10^8 CFU yields similar systemic IgG responses, both in magnitude and kinetics, to those seen when 2×10^6 CFU are inoculated intraperitoneally (i.p.) (Fig. 1a). A 10^6 CFU dose of rBCG-OspA delivered i.n. elicited a comparable immune response to the 10^8 CFU dose over time; however, the time-to-peak response was delayed by 4–6 weeks (Fig. 1b). The anti-OspA IgG antibodies stimulated by i.n. immunization exhibited strong *in vitro* growth-inhibiting titres against *B. burgdorferi* strain B31 (ref. 13) and i.n.-immunized mice were completely protected from infection by the infectious Sh.2 strain of *B. burgdorferi*¹⁴ (Table 1). Intranasal immunization also resulted in a highly sustained, low-titre serum IgA response against OspA (Fig. 1a). Intraperitoneal immunization with 10^6 CFU rBCG-OspA did not result in any measurable serum IgA titre to OspA (Fig. 1). Furthermore, i.n. immunization induced high levels of OspA and BCG-specific IgA spot-forming cells (SFC) in the lungs (Fig. 2), indicative of a strong secretory IgA response^{15–18}. The secretory IgA response could be detected as early as 6 weeks and as late as 22 weeks (the latest time tested) after immunization. Intraperitoneal immunization did not yield

any OspA or BCG-specific IgA SFC, even at a dose of 10^8 CFU.

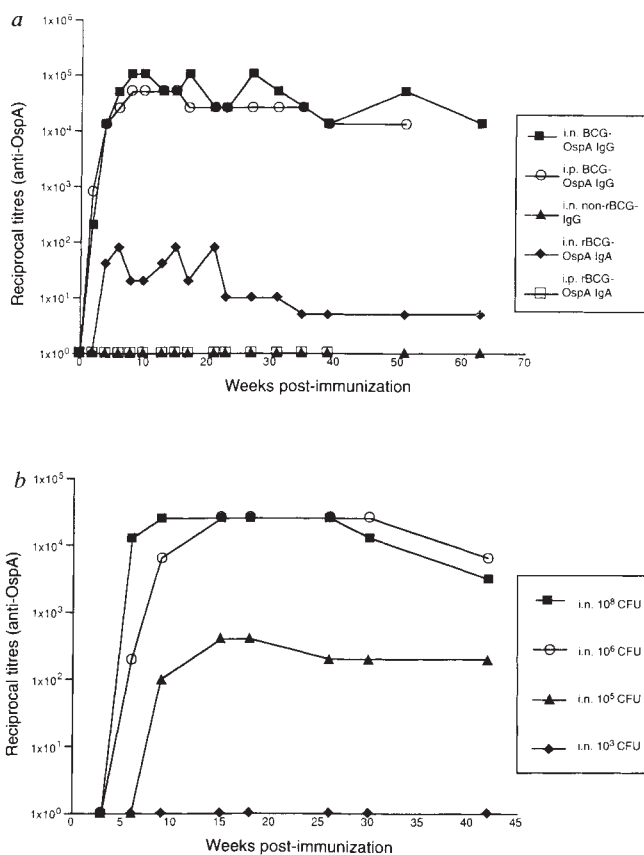
Mononuclear cells isolated from the lamina propria of the gastrointestinal tract of i.n.-immunized mice contained high frequencies of OspA-specific IgA SFC as measured by an ELISPOT assay. Between 10^2 and 10^3 OspA-specific SFC per 10^6 cells were detected at 9 and 16 weeks after immunization (data not shown). No OspA-specific IgA plasma cells were detected in the gut of i.p.-immunized mice. IgA against OspA was also found in vaginal washes of i.n.-immunized, but not i.p.-immunized mice, as early as 6 weeks post-immunization. A total of $0.08 \mu\text{g ml}^{-1}$ of anti-OspA IgA was detected in pooled, vaginal secretions¹⁹ from three i.n.-immunized mice which represented $\sim 8\%$ of the total amount of IgA detectable in the vaginal tract. Antigen-specific vaginal IgA responses persisted for at least 19 weeks.

Unlike i.n. immunization, oral delivery of rBCG (10^7 CFU) in bicarbonate buffer elicited low levels of OspA-specific systemic IgG (end-point titres 1/400); low levels of antigen-specific IgA were found in the gastrointestinal tract, as measured by assaying for antigen-specific IgA in fecal-pellet extracts¹⁹ (data not shown), but not at other mucosal sites (Fig. 2). These low titres may be due to decreased survival of BCG in the gastrointestinal tract; either a tenfold increase in the dose of BCG (10^8 CFU) or pretreatment of mice with the histamine receptor antagonist cimetidine (followed by 10^7 CFU BCG) resulted in a fourfold enhancement in systemic IgG responses (1:6,400).

Microscopic and gross anatomical analysis of lung tissue from i.n.-immunized mice revealed discrete foci of lymphocytic infiltrates (Fig. 3a). These lymphoid aggregates were highly organized in a focal perivascular and peribronchiolar distribution and

FIG. 1 Serum IgG and IgA titres to OspA following intranasal (i.n.) or intraperitoneal (i.p.) immunization with rBCG-OspA or with non-rBCG. **a**, Persistence of serum IgG levels against OspA in BALB/c mice immunized either i.n. or i.p. with the rBCG-OspA vaccine versus persistence of the serum IgA response only in the i.n.-immunized mice. **b**, Dose response for mice immunized intranasally with rBCG-OspA2619 (serum IgG levels). The serum immune responses to OspA were determined for pooled serum samples from five individual mice in each group, every two to four weeks post-immunization, by enzyme-linked immunosorbent assays (ELISAs). Antibody titres shown in **a** were determined up to week 52 (for i.p.-immunized) or week 63 (i.n.-immunized), the latest time points evaluated.

METHODS. ELISA plates (Immulon 1) were coated with $50 \mu\text{l}$ OspA protein ($0.1 \mu\text{g ml}^{-1}$; Connaught, Swiftwater, PA) or $50 \mu\text{l}$ of a 1/500 dilution of whole BCG lysate (10^9 CFU) suspended in 0.1 M carbonate buffer (pH 9.6) and incubated overnight at 4°C . The antigen solution was removed and plates were incubated with blocking solution (0.5% BSA and 0.5% non-fat dry milk + 0.2% sodium azide in H_2O) for 1 h at room temperature. Twofold serial dilutions starting at either 1:10 (IgA assays) or 1:200 (IgG assays) of pooled serum from five individual BALB/c mice in each group, were prepared in blocking solution and $50 \mu\text{l}$ of each dilution was added to duplicate wells of the antigen-coated plate and incubated at room temperature for 1 h; for all groups in **a** and **b**, the standard error of the mean titre for individual mice within a group did not exceed 4.3%. Plates were then washed with PBS + 0.1% Tween-20 (PBS-T20) and incubated with $50 \mu\text{l}$ of a 1:4,000 dilution of peroxidase-conjugated goat anti-mouse IgG or IgA (Southern Biotechnology Associates) for 1 h. Plates were developed with 2, 2'-azino-di[3-ethyl-benzthiazoline sulphinate (6)] (ABTS) substrate reagent (Kierkegaard and Perry Labs) and absorbance was measured at 405 nm (A_{405}) on a Dynatech ELISA reader. Endpoints were defined as the highest dilution at which the A_{405} values were twice the values for preimmune sera diluted 1:10 or 1:200 in blocking solution. Serum from mice immunized with non-rBCG did not react with the OspA antigen, as shown. Intranasally immunized mice were inoculated with a dose of 1×10^8 CFU rBCG-OspA2619S or non-recombinant BCG in $50 \mu\text{l}$ PBS on 2 consecutive days (total dose, 2×10^8 CFU). Intraperitoneally immunized mice received a total of 2×10^6 CFU in $100 \mu\text{l}$ PBS. In the rBCG vaccine strain (rBCG-2619S/OspA) used in these experiments, OspA is expressed as a surface-associated lipoprotein similar to the rBCG-p19PS/OspA described earlier¹⁰, except that OspA expression in rBCG-2619S/OspA is driven by the mycobacterial *hsp60* promoter. Only BALB/c mice were



included in these immunogenicity studies on the basis of a previous survey of immune responsiveness to OspA among 24 inbred and outbred mouse strains¹⁰.

TABLE 1 *In vitro* growth inhibition titres and protection from intradermal challenge with *B. burgdorferi*

rBCG vector/route of immunization	Growth inhibition titre	Plasma culture(+)/total	Heart culture(+)/total	Bladder culture(+)/total	Tibiotarsal joint culture(+)/total
rBCG-OspA/i.n.	1:16,384	0/7	0/7	0/7	0/7
rBCG-OspA/i.p.	1:32,768	0/7	0/7	0/7	0/7
Non-rBCG/i.n.	<1:8	3/7	3/7	7/7	6/7
Non-immunized	1:64	1/7	3/7	7/7	6/7

In vitro growth inhibition titres against the B31 strain of *B. burgdorferi* were determined as described¹¹ using pooled sera from mice immunized i.n. or i.p. with rBCG-OspA, mice immunized i.n. with non-rBCG, or non-immunized mice. 96-well, flat-bottom plates were seeded with 2×10^6 spirochetes in 0.1 ml BSK-II medium derived from late log-phase culture of a high-passage isolate of *B. burgdorferi* B31 (ATCC35210) grown in the same medium. Twofold serial dilutions were added to duplicate wells, and the plates incubated at 34 °C for 72 h in the presence of 1% CO₂. Bacterial growth was monitored by colour changes in phenol red indicator dye. Growth inhibition titres shown are complement-dependent; complement-independent titres for mice immunized i.n. or i.p. with rBCG-OspA were 1:3,200 and 1:6,400 respectively. For challenge experiments, immunized and age-matched, non-immunized control BALB/c mice were challenged intradermally (i.d.) at the base of the tail, 13 weeks after immunization, with 10^4 spirochetes (~100 ID₅₀ units) derived from the low-passage Sh.2 strain of *B. burgdorferi*¹²; there were 7 mice per group. Mice were killed 14 days after challenge and their hearts, bladders and tibiotarsal joint tissues were minced and cultured together with blood samples from each animal in BSK-II medium. Cultures were monitored up to day 14 by phase-contrast microscopy for the presence of spirochetes. The presence of even a single spirochete in any of twenty fields was scored as a positive infection. Challenge experiments done as late as 25 and 40 weeks post-immunization also demonstrated sterile immunity in rBCG-OspA-immunized mice (G. Bansal, MedImmune). Data are presented as number of mice giving positive cultures at a particular site per total mice in the group; some of the mice in the non-immunized and control non-rBCG groups had positive cultures at more than one site, and all control mice had positive bladder cultures.

contained a mixture of monocytes, macrophages, activated lymphocytes and plasma cells (Fig. 3*b, c*). The appearance of lymphoid aggregates correlated with the emergence of systemic and local immune responses to rBCG. The lymphoid aggregates persisted at least 22 weeks after immunization. Despite vigorous recruitment of immune cells into the lungs of i.n.-immunized mice, there was no evidence of fibrosis or granuloma formation over a 22-week period.

Flow cytometric analysis (fluorescence-activated cell sorting, FACS) of 10^6 intraparenchymal lung lymphocytes from i.n.-immunized, i.p.-immunized or naive mice revealed a threefold increase of B cells in the lungs of mice immunized i.n. with either rBCG or BCG lacking the recombinant vector, compared to lungs isolated from i.p.-immunized mice or naive mice (data not shown). Periodic acid-Schiff base staining showed a significant number of plasma cells, consistent with the FACS analysis. FACS revealed no significant increases in the percentage of CD4⁺ and CD8⁺ cells in the lungs of i.n.-immunized mice relative to naive or i.p.-immunized mice (~25 and 5–10%, respectively, in all cases). No differences were noted in the CD4/CD8 ratios among the different groups.

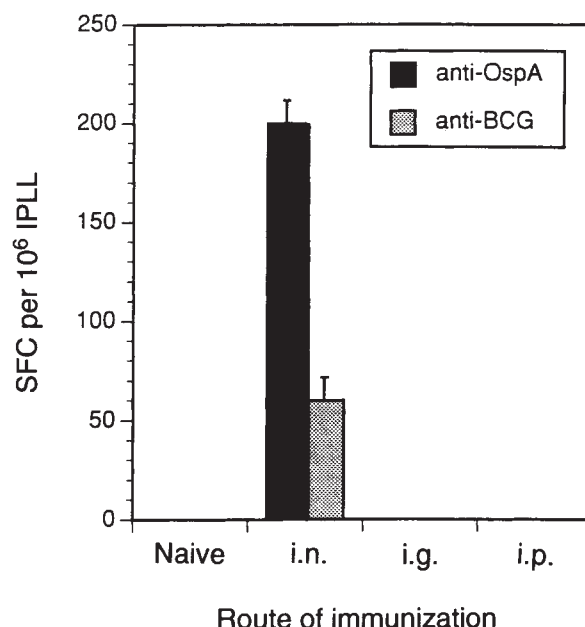
FIG. 2 Secretory immune response to OspA occurs in respiratory tract only when mice are immunized i.n. as determined by an ELISPOT assay. Results are expressed as the mean number $\pm$ s.e.m. of spot-forming cells (SFC/IgA-producing) per 10^6 cells (intraparenchymal lung lymphocytes (IPLs)), the limit of detection of the assay. i.n., intranasal; i.g., intragastric; i.p., intraperitoneal.

METHODS. The ELISPOT assay was used to determine the number of cells spontaneously secreting antigen-specific IgA tissues of mucosally immunized mice (secretory immune response)^{23,24}. 96-well flat-bottom plates with a nitrocellulose base (Millititer-HA, Millipore) were coated overnight at 4 °C with either OspA protein ($0.1 \mu\text{g ml}^{-1}$) or a 1/500 dilution of whole BCG lysate in PBS (100 μl per well). Plates were washed with PBS ($\times 3$) and incubated for 1 h with blocking solution (RPMI 1640 + 10% fetal calf serum). After removal of the blocking solution, lymphocytes isolated from lung tissue of a total of 3 mice per group were pooled and added at varying concentrations ranging from 1×10^2 – 1×10^5 lymphocytes per well, and cultured overnight at 37 °C in air with 5% CO₂ and 95% humidity. The IPLs were isolated from mice 22 weeks after immunization by standard protocols¹⁷. After overnight incubation, plates were washed with PBS ($\times 3$) and PBS + 0.05% Tween ($\times 3$) before addition of 100 μl of a 1:500 dilution of peroxidase-conjugated goat anti-mouse IgA (SBA) for 1 h. Plates were developed with 3-amino-9-ethylcarbazole substrate reagent (Pierce). Red-brown-coloured spots were counted with the aid of a dissecting microscope (Zeiss). The lymphocyte isolation method consistently yielded ~ 1 – 2×10^6 cells per mouse with >98% viability.

Peyer's patches isolated from the gastrointestinal tract of i.n.-immunized mice revealed lymphoid accumulations in follicles underlying the domed epithelium which were grossly larger than lymphoid aggregates seen in Peyer's patches of unimmunized or i.p.-immunized mice (data not shown). Lymphocytic infiltrates were also seen in the lamina propria and muscularis mucosa of i.n.-immunized mice, and large numbers of lymphocytes were seen extending into the villi as well.

Dense foci of lymphocytes were also observed in the nasopharyngeal-associated lymphoid tissue (NALT) of i.n.-immunized mice. Comparable lymphoid aggregates were absent in unimmunized mice or mice immunized i.p. (Fig. 3*e–g*). The appearance of organized lymphoid cell accumulations in NALT correlated with the appearance of a secretory immune response to the OspA antigen. The induction of organized lymphoid aggregates in NALT suggests a role for NALT in the generation of systemic and secretory responses against inhaled antigens.

At this point it is uncertain whether the appearance of lymphocytic foci in NALT and in distal tissues is due either to uptake of BCG by NALT microfold-cell equivalents followed by dissemination of BCG to distal tissues, or to stimulation of



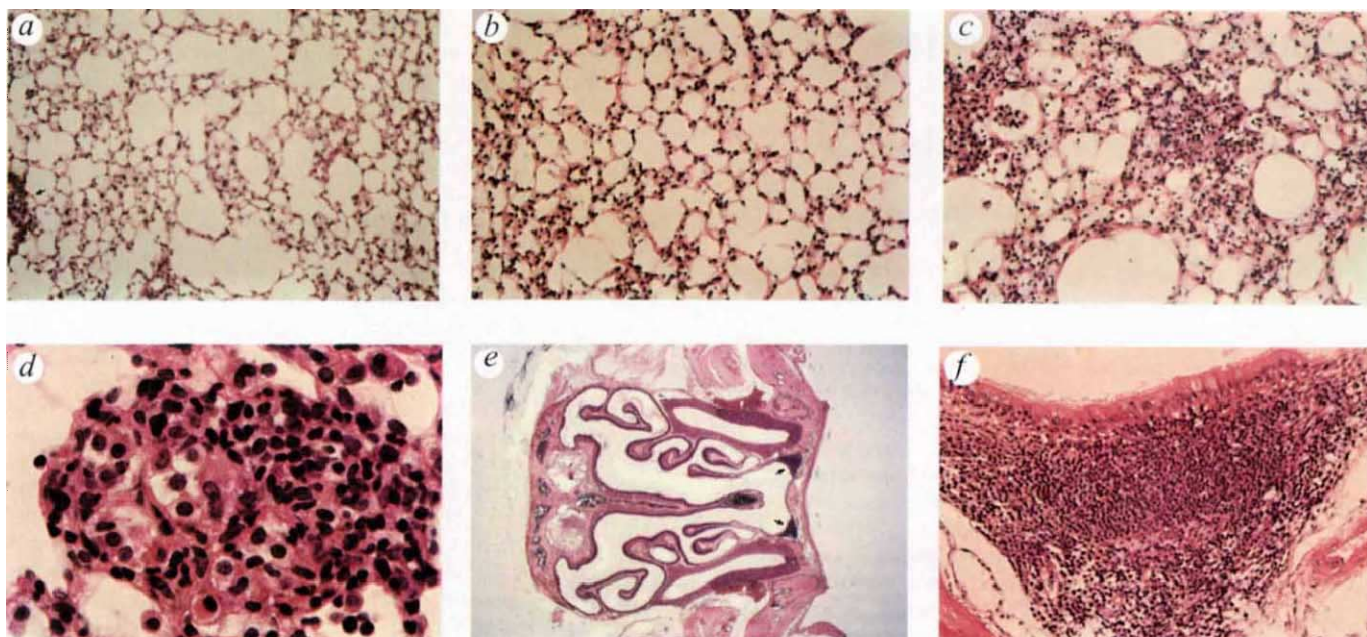


FIG. 3 Mouse lung and nasopharyngeal lymphoid tissue sections stained with haematoxylin and eosin. *a*, Section taken 6 weeks after i.n. immunization with 10^8 CFU rBCG–OspA. Note regions of significant peribronchial lymphocytic infiltrates at the terminal bronchi (arrow) in otherwise normal-looking lungs. Magnification, $\times 76$. Similar histological changes were seen in mice immunized i.n. with non-recombinant BCG. *b*, Section taken 6 weeks after i.p. immunization with 10^8 CFU rBCG–OspA. No regions of localized lymphocytic infiltrates were seen. Sections from non-immunized mice appeared to be the same as those taken from the i.p.-immunized mice. Magnification, $\times 76$. Close-up views of *c*, peribronchial and periarterial lymphocytic infiltrates seen in the lungs of i.n.-immunized mice (magnification, $\times 190$), and *d*, one of the lymphoid aggregates (magnification, $\times 380$). Note the diverse population of lymphoid cells including monocytes, macrophages and lymphocytes within the aggregate. *e*, Cross-section through mouse nasal passages, at the level of the ecto- and endoturbinates^{25,26}, revealing bipolar distribution of nasopharyngeal lymphoid tissue (NALT; arrows); magnification, $\times 7.6$. *f*, Section of NALT from non-immunized mouse (magnification, $\times 190$). *g*, Section of NALT taken 6 weeks after i.n. immunization with 10^8 CFU rBCG–OspA (magnification, $\times 95$). Note the dense accumulation of highly organized lymphoid tissue (arrow).

lymphocytes in the nasal mucosa followed by recirculation and homing to other mucosal tissues. Acid-fast and auromine-O staining of serial tissue sections from nasopharyngeal-, bronchial- and gut-associated lymphoid tissue as well as from spleen and liver tissue failed to reveal any BCG at these sites. However, quantitative culturing of lung and spleen tissue from i.n.-immunized mice has shown BCG persistence in lungs and spleen at least 9 weeks post-immunization. Conversely, although BCG was found in both spleens and lungs of i.p.-immunized mice at 1 day, and at 2 or 4 weeks post-immunization, rBCG–OspA was isolated only from spleens of i.p.-immunized mice at 9 weeks post-immunization. This finding suggests that persistence of BCG at proximal and distal sites of the mucosal immune system may be responsible in part for the sustained local immunity. Whether the NALT is also a primary site for stimulation of antigen-specific lymphocytes which then home to distal mucosal sites (for example, the gastrointestinal and genitourinary tracts)^{20–22}, as well as to the spleen, is being investigated.

We conclude that intranasal delivery of rBCG–OspA results in a potent, long-lasting systemic antibody response and a strong, pan-mucosal secretory IgA response to the vaccine. Although OspA was chosen as a model antigen to evaluate induction of immune responses following mucosal delivery of rBCG, we recognize that *B. burgdorferi* does not infect through a mucosal surface. Nonetheless, our findings are significant in that they demonstrate that i.n. immunization with rBCG can elicit complete protection against systemic infection by the native organism expressing that same antigen, while inducing a sustained mucosal immune response. It remains to be determined

whether mucosal immunization with rBCG expressing an antigen from a mucosal pathogen can protect against infection at a mucosal site. □

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Locus-specific somatic hypermutation in germinal centre T cells

Biao Zheng, Wei Xue & Garnett Kelsoe

Department of Microbiology and Immunology, University of Maryland School of Medicine, 655 West Baltimore Street, Baltimore, Maryland 21201, USA

SOMATIC hypermutation and affinity-driven selection of active immunoglobulin genes occur in germinal centres (GCs), resulting in the generation of high-affinity memory B cells¹⁻³. In contrast, T lymphocytes do not require the germinal centre microenvironment to establish memory⁴ and the T-cell antigen receptor (TCR) genes, though homologous to immunoglobulin genes, are believed to be incapable of hypermutation⁵⁻⁷. Here we present direct evidence that the small population of antigen-specific T cells that are recruited into splenic GCs acquire mutations in the variable region of genes encoding TCR α -chains ($V\alpha$) but not those of β -chains. These locus-specific mutations reach frequencies comparable to mutated immunoglobulin V_H exons recovered from the same site and exhibit similar substitution biases and DNA strand polarity. T cells bearing identical mutations appear in multiple GCs, raising the possibility that some cells bearing mutant TCRs may re-enter the peripheral lymphocyte pool.

Although most immune responses are mediated by diverse populations of lymphocytes, the T and B cells of B10.A mice ($H-2^k; IgH^b$) that react to pigeon cytochrome *c* (PCC) substituted with the (4-hydroxy-3-nitrophenyl)acetyl hapten (NP) are clonally restricted. More than 70% of the $CD4^+$ T cells that recognize PCC bear TCR encoded by the $V\beta 3$ and $V\alpha 11$ gene families⁸. Likewise, >90% of NP-reactive B cells express

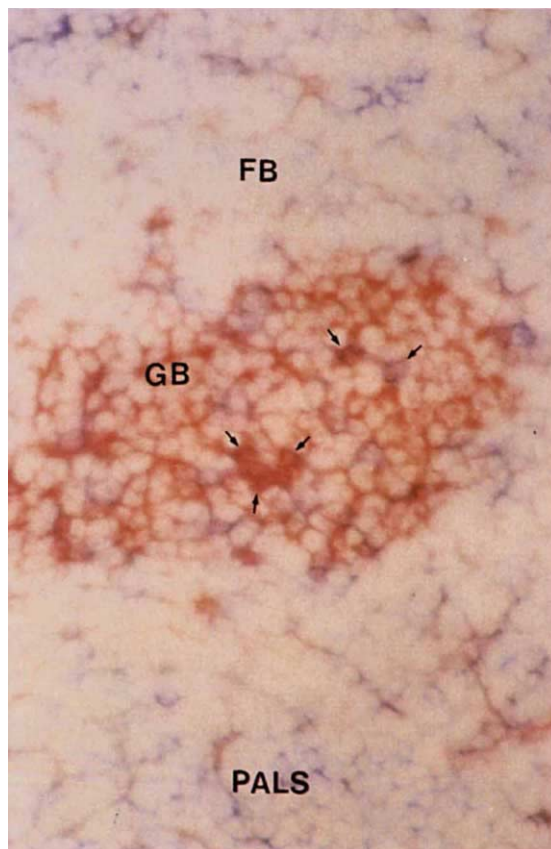
immunoglobulins encoded by the V186.2 heavy-chain and $V\lambda 1$ light-chain gene exons^{9,10}. These populations can be identified by immunohistology^{11,12} and the somatic genetics of TCR and immunoglobulin genes within them studied by microdissection of labelled cells from sections and amplification of the recovered DNA by the polymerase chain reaction (PCR)^{3,13,14}.

After immunization with PCC-NP, activated $V\alpha 11^+$ T cells and $\lambda 1^+$ B cells can be identified in two major sites within the spleen, the periarteriolar T-cell sheath (PALS) and the GC (Fig. 1). Numbers of $V\alpha 11^+$, $CD4^+$ cells rapidly increase in the PALS in the first 4 days of the response and then decline in association with migration to other sites including the B-cell follicles and nascent GCs (refs 12, 15, 16 and our unpublished results). Sparse foci of NP-binding, $\lambda 1^+$ plasmacytes are present in the PALS by day 6 and GCs that bind the lectin, peanut agglutinin (PNA), first appear 8 days postimmunization. The magnitude and kinetics of these responses suggest that PCC is less immunogenic than some other carriers (refs 3, 11-13 and our unpublished results).

Rearranged $V\alpha 11$ ($V\alpha 11/J\alpha 84$), $V\beta 3$ ($V\beta 3/J\beta 1.2$), V_H (V186.2/ $J_H 2$), and $V\lambda 1$ ($V\lambda 1/J\lambda 1$) genes¹⁷⁻²¹ were recovered in samples of 20-40 cells from the PALS and GCs of immune and naive mice, amplified by the *Pyrococcus furiosus* (*Pfu*) polymerase²² and ligated into plasmid vectors for bacterial transformation^{3,13}. $V(D)J$ inserts from single bacterial colonies were sequenced and compared to the germ-line $V\alpha 11$, $V\beta 3$, $V_H 186.2$ and $V\lambda 1$ genes. In some instances, DNA recovered from congruent sites in adjacent tissue sections was amplified and sequenced independently to control for PCR artefacts^{3,12,13}.

Table 1 summarizes our study of mutations in 104 kilobases (kb) of $V(D)J$ DNA from antigen-specific T and B lymphocytes from B10.A mice. A control value for PCR error was established by amplifying and sequencing the rearranged $V\alpha 11.1$ exon of the B10 cell line²³, yielding a background frequency of 0.7×10^{-4} misincorporations per base pair (bp). Frequencies of misincorporations in $V\alpha 11$ genes from the PALS (2.1×10^{-4} bp⁻¹) or

FIG. 1 Histological demonstration of antigen-specific recruitment of $V\alpha 11^+$, $CD4^+$ T cells to PNA⁺ GCs induced by immunization of B10.A mice with NP-PCC (200X). Triple colour immunostaining identifies splenic cells expressing $V\alpha 11^+$ TCR (stained pink), $CD4^+$ cells (stained blue), and GC B cells (stained red). At day 12 of a primary response, the PALS contains many $CD4^+$ cells but relatively few (6%-11%, unpublished data) express $V\alpha 11^+$ TCR. In contrast, the majority (55%-95%) of $CD4^+$ GC cells coexpressed $V\alpha 11$ (arrows). These double-positive T lymphocytes are scattered among the PNA⁺ B cells of the GC light zone (GB). Follicular B lymphocytes not involved in the GC reaction are unlabelled (FB). This spleen was taken from a B10.A mouse immunized 12 days earlier with 50 μ g NP-PCC in alum. Serial, 6- μ m-thick sections were cut from the frozen spleen, thaw-mounted onto poly-L-lysine coated slides, fixed in cold acetone, and stored before use at -80°C ^{3,11}. For three-colour labelling, sections were incubated with the rat anti- $CD4$ antibody, GK1.5 and with PNA coupled to horseradish peroxidase (PNA-HRP). This incubation was followed by goat anti-rat immunoglobulin linked to alkaline phosphatase (GaRlg-AP). After washing, sections were further incubated with biotinylated RR8-1 antibody (Pharmingen) specific for $V\alpha 11^+$ TCR. Specificity of the RR8-1 antibody was confirmed by staining $V\alpha 11^+$ (B10) and $V\alpha 11^-$ (HDK-1, EL-4 and P3) cell lines. Bound AP and HRP were then visualized using naphthol AS-M phosphate/fast blue BB and 3-aminoethyl cabazole, respectively. Slides were then treated in 1M HCl for 15 min, rinsed and incubated with streptavidin-AP. Second round AP activity was detected with fast red.



V β 3 from GCs ($<0.5 \times 10^{-4}$ bp $^{-1}$) did not significantly differ ($P>0.05$) from this background. However, a significant excess of mutations was observed in V α 11 gene segments recovered from the GCs of immunized mice. A total of 61 mutations was present in 42,840 bp yielding an error frequency (14.2×10^{-4} bp $^{-1}$), roughly 15 times greater than PCR error.

Amplification of the first exon of the TCR α -chain constant region gene (C α (1)) from the same GCs demonstrated that these excess mutations were confined to the V-region (Table 1). GCs that contained mutated V α 11 genes also contained comparably mutated V H gene segments. Eighteen V H 186.2 rearrangements recovered from 3 GCs, contained 27 mutations for a frequency

	8	12	16	23	27	32	35	39	50	70	77	82	83	89	90	N Region	Ja84
	P	S	G	C	T	V	F	S	A	S	A	S	G	A	E		
V α 11.1	CCT	AGC	GGA	TGC	ACC	GTG	TTC	TCC	GCT	AGC	GCC	TCA	GGC	GCT	GAG		CCT TCA AGT GGC
V α 11.2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---		
V α 11.3	---C	---T	---	---	---T	---	---	---C	---TG	---	---	---	---	---	---		
DAY 12																	
D12GC1.II.2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---G	g g g g g *	g ---
D12GC1.I.1	---	---G	---	---	---	---	---	---	---	---	---	---	---	---	---G	g c g g g *	g ---
D12GC1.I.5	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---G	g c g g g *	g ---
D12GC1.II.1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---G	g c g g g *	g ---
D12GC1.II.5	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---G	g c g g g *	g ---
D12GC1.I.9	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---G	g c g g g *	g ---
D12GC3.3	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---G	g c g g g *	g ---
D12GC3.7	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---a	g c g g g *	a ---
D12GC3.9	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---a	g c g g g *	a ---
D12GC4.4	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---a	g c g g g *	a ---
D12GC4.3	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---a	g c g g g *	a ---
D12GC4.6	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---a	g c g g g *	a ---
D12GC4.11	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---A	g c g g g *	a ---
D12GC5.I.5	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---G	g c g g g *	g ---
D12GC5.II.11	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---G	g c g g g *	g ---
D12GC5.II.2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---G	g c g g g *	g ---
D12GC5.II.3	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---G	g c g g g *	g ---
D12GC5.I.13	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---G	g c g g g *	g ---
D12GC5.I.8	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---G	g c g g g *	g ---
D12GC5.I.9	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---G	g c g g g *	g ---
D12GC5.I.11	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---G	g c g g g *	g ---
D12GC5.I.1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---G	g c g g g *	g ---
D12GC5.I.6	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---G	g c g g g *	g ---
D12GC5.II.1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---G	g c g g g *	g ---
D12GC6.8	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---C	g c g g g *	a ---
D12GC7.10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---c	g c g g g *	a ---
D12GC22.8	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---c	g c g g g *	a ---
D12GC22.2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---c	g c g g g *	a ---
DAY 16																	
D16GC1.4	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	g *	l c a a ---
D16GC1.6	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	g *	l c a a ---
D16GC1.11	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	g *	l c a a ---
D16GC1.2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	a ---
D16GC1.3	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	a ---
D16GC3.5	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	a ---
D16GC3.8	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	a ---
D16GC3.2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	a ---
D16GC3.6	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	a ---
D16GC3.7	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	a ---
D16GC3.9	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	a ---
D16GC3.11	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	a ---

FIG. 2 The 39 mutated V α 11 sequences recovered from V α 11 $^{+}$ GC cells 12 and 16 days after immunization with NP-PCC. Mutated V α 11/J α 84 rearrangements were recovered from 3 of 4 mice. The GCs, D12GC1, -3, -4, -5, -6 and -7; D12GC22; and D16GC1 and -3 represent individual spleens. Codons that differ between the V α 11.1, -11.2 and -11.3 homologues (top) and that contain mutations are illustrated. The amino acids specified by the V α 11.1 gene segment are given using the single-letter code. Sequences are organized by date (day 12, 16), GC number, and sequence number (such as D12GC3.3). Sequences indicated with the prefixes I or II are derived from independent PCR amplifications of the same GC taken from two adjacent serial sections. Codons are numbered according to ref. 17. Dashes indicate identity to the reference V α 11.1 sequence, slashes indicate a deletion, misincorporations are identified in italics. Mutations leading to amino-acid replacements are marked with dark circles defining the substitution by the single-letter code. Nonproductive V α 11.1/J α 84 joints are indicated with asterisks. Junctional sequences are indicated by the lower case. A total of 61 mutations were observed, broadly distributed throughout the V α 11.1 gene segment. A single independent parallel mutation was seen, 82TCA $\rightarrow$ TCT in D12GC3.9 and in those sequences related to D12GC3.3. D12GC4.11 shares a 90GAG $\rightarrow$ GAA mutation identical to

junctional diversity (90GAA) present in D12GC4.4. It is possible that the D12GC3.9 and D12GC4.11 sequences represent PCR hybrids; no other mutations could have resulted by this mechanism. Two mutations in D16GC3 may be presumed to be deleterious: a deletion in codon 16 resulting in frame-shift and a replacement of the invariant Cys at position 23. The observed ratio of replacement to silent (R:S) unique mutations is 2.2:1, below that expected (3.3:1) for random mutagenesis. The ratios of P:nP genotypes may be used to estimate independently the frequency of V α 11 $^{+}$ cells within an excised sample. Consider any rearranging locus that does not exhibit allelic exclusion. If cells exhibiting the nP/nP genotype are lost, the expected ratio of P and nP alleles in the absence of selection is 1.5P:1nP. Higher ratios indicate phenotypic enrichment whereas lower ratios suggest selection against the expressed phenotype. Assuming an independent probability of 0.1 for V α 11 rearrangement (the frequency of V α 11 $^{+}$ CD4 $^{+}$ T cells, not shown) the average P:nP ratio for unmutated V α 11 rearrangements from GC, 7.7:1, correspond to a T-cell population that is $\sim$ 60% V α 11 $^{+}$, confirming our histological determinations (Fig. 1). In contrast, the ratio of 0.9:1 found in mutated V α 11 rearrangements suggests that only about 6% of this population expressed V α 11 $^{+}$ TCR; 54% of the mutated V α 11 gene segments functioned as non-selected, passenger genes 27 .

of $53.8 \times 10^{-4} \text{ bp}^{-1}$. Analysis of *V λ 1* genes revealed no excess mutations (not shown). Mutated *V λ 11* exons contained slightly fewer substitutions (average, 1.6; range, 1–3) than did mutated *V $\text{H}186.2$* segments (average, 2.1; range, 1–5).

All mutated *V λ 11* sequences are shown in Fig. 2, arranged by their date and site of recovery. Twenty unique mutations (19 nucleotide substitutions, 1 deletion) were uniformly distributed with misincorporations equally divided between transitions (10) and transversions (9). Both productive (P) and out-of-frame (nP) *V λ 11* rearrangements were recovered from the PALS and GCs of immune mice, with P rearrangements more than twice as common in GC versus the PALS on day 12 and 16 of the response. The P:nP ratio for all GC *V λ 11* rearrangements was 3.7:1 (134:36). However, only 18 of the 39 mutated *V λ 11* rearrangements (Fig. 2) represented P VJ joints, for a P:nP ratio of 0.9:1. A much higher ratio, 7.7:1 (116:15), characterized unmutated GC *V λ 11* rearrangements. As the TCR α loci do not exhibit allelic exclusion²⁴, ratios of P:nP *V λ 11* genotypes predictably reflect the frequency of *V λ 11*⁺ cells (see legend to Fig. 2). Ratios observed in GC predict T-cell populations containing 30% to 70% *V λ 11*⁺ cells. These estimates confirm histological enumeration (Fig. 1 and ref. 15). Bias for mutations in nP templates probably reflects intense phenotypic selection against expressed mutant TCR and is inconsistent with abiotic processes, such as polymerase errors or generation of chimaeric sequences^{13,22,25}. In mutated *V λ 11* genes, ratios of replacement to silent mutations were similar in both P and nP rearrangements (Fig. 2). However, mutations in *V λ 11.1* were not random, exhibiting a marked bias for A→G transitions (4/19 mutations) with A→N exchanges ($n=7$) more than twice as common as the reciprocal T→N ($n=3$) events. This pattern of biased nucleotide

substitution and DNA strand polarity is characteristic of immunoglobulin hypermutation^{26,27}. Indeed, the observed mutations differ substantially ($\chi^2=4.25$; d.f.=3) from unselected meiotic point mutations but are indistinguishable ($\chi^2=0.02$; d.f.=3) from mutations flanking the coding regions of hypermutated immunoglobulin genes²⁷.

Mutated *V λ 11* genes were not uncommon. Nine of 17 *V λ 11* rearrangements recovered from GCs contained point mutations; two-thirds of the GCs sampled (11/16) contained mutated *V λ 11.1* sequences. Thus, TCR α -chain mutations occur at significant rates only in the GC microenvironment, are limited to the V-region, share the character of immunoglobulin hypermutation, and are present at frequencies comparable to mutated immunoglobulin V genes recovered from the same site.

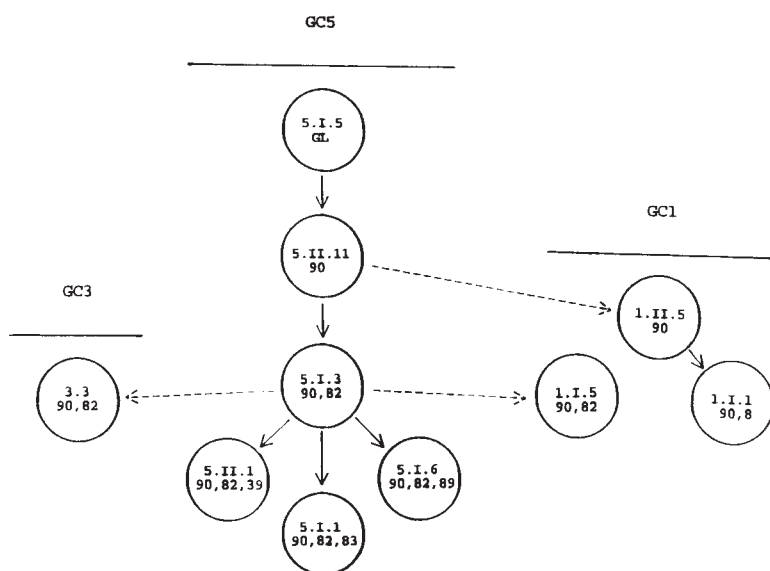
Mutant TCR genes are not polymerase artefacts. Introduction of nucleotide error by the *Pfu* polymerase is rare and the probability of observing $n=1, 2, 3$ and so on, misincorporations in an amplified template should follow a Poisson distribution^{11,13}. Only the distribution of mutations in *V λ 11* and *V $\text{H}186.2$* rearrangements from GCs differed significantly ($P<0.001$) from the expected distribution (not shown). Another common artefact of the PCR is generation of hybrids during the amplification of similar DNA templates^{3,13}. This artefact is not responsible for the mutations we have observed. (1) No mutation could result from hybrids formed between the three *V λ 11* homologues known in the B10.A strain (Fig. 2 and refs 17, 23, 28). (2) *V β 3* rearrangements were unmutated. Hybrids should be equally common in amplifications of the α and β loci. (3) Each GC sample contained 4–10 *V λ 11*⁺ cells and no more than 7 unique VJ products arose in any PCR (Fig. 2 and data not shown). Frequent hybrids would have resulted in excess VJ sequences¹³.

TABLE 1 Mutations in TCR and immunoglobulin variable- and constant-region genes recovered from different histological sites

Cell source (no. of samples)	Type/no. genes recovered	No. base pairs sequenced	No. unique V(D)J joints	No. observed mutations	Frequency of observed mutations ($\times 10^{-4}$)
B10 line	<i>Vλ11/56</i>	14,112	1	1	0.7
PALS ($n=12$)	<i>Vλ11/134</i>	33,768	23	7	2.1
GC ($n=7$)	<i>Vβ3/82</i>	21,894	4	0	<0.5
GC ($n=16$)	<i>Vλ11/170</i>	42,840	17	61	14.2
GC ($n=4$)	<i>Cα(I)/46</i>	10,350	—	0	<1.0
GC ($n=3$)	<i>v$\text{H}186.2/18$</i>	5,022	9	27	53.8

Mutations in TCR and immunoglobulin variable- and constant-region genes recovered from different histological sites. Female B10.A mice were immunized with 50 μg NP-PCC in alum^{11,12}. Groups of 2–3 mice were killed at various times postimmunization and their spleens processed for histology. *V λ 11*⁺ cells within PNA⁺ GC were identified by specific immunolabelling (Fig. 1) and recovered from tissue sections by microdissection^{3,13,14}. Excised cell matter was incubated with proteinase K overnight at 37 °C and after heat-inactivation of the proteinase at 96 °C, the crude lysate was subjected to two rounds of PCR amplification. PCR primers used in this study are given in the format: 5'-external/3'-external; 5'-internal/3'-internal. Primers used to amplify *V $\text{H}186.2/J μ 2$* rearrangements have been described^{3,13}. *V λ 11/J λ 84*: 5'-GTGCTGGGGATTCTGTGGGTGCAGATTGCTG-3'/5'-CAGGTACACCTTTAATATGGTCCCTGGCCAAA-3'; 5'-CTGCAGGAGATCAGGTGGAGCAG-3'/5'-ATGGATCCAAAACACAGCTTCTGGCC-3'. *V β 3/J β 1.2*: 5'-TACACAGTACTTTGTCTCCTGGGTGCAAGAAT-3'/5'-TATTACCAAAAGCCTGGTCCCTGAGCCGAAG-3'; 5'-AGCTGCAGAATTCAAAAGTCATTGAGACT-3'/5'-ATGGATCCTGAGCCGAAGGTGTAGTC-3'. *C α (I)*: 5'-CATCAGCCTCTTGGCCACAGACATCCAGAACCCA-3'/5'-TTCTGGGGCACCCTACCTGAAGTGGGTAGGT-3'; 5'-CTGCAGACATCCAGAACCCAGAA-3'/5'-ATGGATCCCTGAAGTGGGTAGGTGGC-3'. *V λ 11/J λ 1*: 5'-ACTCTCTCTCCTGGCTCTCA-3'/5'-GCACCTCAAGTCTTGGAGAGAG-3'; 5'-CTACTGTCAGTGGGTATGCAACAATGCG-3'/5'-ACCTAGGACAGTCAGTTTGG-3'. Amplification of template DNA was by the *Pfu* polymerase using nested primers specific for particular V or C exons; each round consisted of 40 cycles at 94 °C for 1 min, 58 °C for 40 s and 72 °C for 1 min. On average, about 60% of samples containing well labelled *V λ 11*⁺ cells gave strong positive PCR results. In contrast, only about 7% of unstained or equivocally labelled GC samples yielded PCR products (data not shown). Amplified DNA fragments were ligated into the pBSK⁺ plasmid vector and cloned by bacterial transformation^{3,13}. Positive bacterial colonies were identified by hybridization and the cloned inserts sequenced in both directions using the Sequenase Version 2 kit or by an Applied Biosystems 373A DNA Sequencer using the Ready Reaction Dyedexy Terminator Cycle Sequencing kit. All mutated sequences were confirmed by blind automated sequencing. Mutation in GC *V λ 11* rearrangements remained comparable to *V $\text{H}186.2$* and significantly above the background of *Pfu* error even if calculated on the basis of unique mutation, that is, shared mutations scored as one event. Calculated in this way, the mutation frequency of *V $\text{H}186.2$* falls 2.3-fold, from 54×10^{-4} to 24×10^{-4} ; the mutation frequency in *V λ 11* is similarly reduced (2.8-fold), from 14×10^{-4} to 5×10^{-4} . Another unbiased estimate of mutation frequencies is to count mutations within specific *V λ 11/J λ 84* rearrangements characteristic of the anti-PCC response. The VJ joint found in the PCC-specific B10 cell line (-GAG GCA ACT-; ref. 23) occurs 11 times in the 170 *V λ 11* fragments recovered from GC. Four of those sequences (D12GC1.9, D12GC4.3, D12GC4.6 and D12GC4.11) contain single, unique mutations suggesting a mutation frequency of $(4/2,772) = 1.44 \times 10^{-4}$. This value is in excellent agreement with the previous estimates and may be compared directly to the error frequency observed in amplification of DNA from the B10 cell line. Mutated *V λ 11* genes are unlikely to represent anomalous *V λ 11/J λ 84* rearrangements in GC B cells because: (1) complete TCR rearrangements have never been found in B lymphocytes³⁰; (2) *V λ 11* amplification was strongly correlated with the presence of labelled *V λ 11*⁺ cells (above); (3) recovery of identical *V λ 11/J λ 84* joints in widely separated GC is inconsistent with the behaviour of GC B cells^{3,11,13}.

FIG. 3 Mutation within a single T-cell clone during the immune response. At day 12 of the immune response to NP-PCC, $V\alpha 11.1/J\alpha 84$ rearrangements with the nP joint V-GCG GG* G-J (all sequences from Fig. 2) were independently recovered from three separate GC, GC5, GC1 and GC3. Sequences prefixed by I or II represent independent amplifications of DNA recovered from adjacent tissue sections; five independent PCR reactions are represented. Active mutation and proliferation may be followed in GC5 as the stepwise accumulation of mutations in codons 90, 82, 39, 83 and 89. This single lineage was recovered from independent PCR samples (I and II) excluding an *in vitro* origin for these misincorporations^{3,11,13}. Although the possibility of independent parallel mutations at positions 90 and 82 cannot be ruled out, the probability of at least 3 identical, independent mutations taking place in the same VJ rearrangement is very small. Thus, cells identical to the 5.II.11 and 5.I.3 lymphocytes may have migrated (dotted arrows) from GC5 to GC1 and GC3. T-cell migration from the PALS to establish multiple GC has been confirmed by the analysis of VDJ joints in amplified $V\beta 3/J\beta 1.2$ rearrangements (not shown) and is distinct from the restricted distribution of V_H rearrangements in the same GC (not shown and refs 3, 13). The latter observation precludes sample contamination.



(4) Genealogies consistent with the iterative accumulation of mutations were observed (Fig. 3). Figure 3 represents five independent PCR amplifications of GC samples taken from adjacent sections of a single spleen. These amplifications produced a single genealogy indicating that the mutations represent a population of related cells rather than the accumulation of artefacts^{3,13}.

To ensure that unknown $V\alpha 11$ elements were not confused for mutant $V\alpha 11.1$ genes, DNA from the liver of B10.A mice was amplified (40 cycles) with *Pfu* polymerase using the standard internal 5' primer for $V\alpha 11$ (Table 1) and a 3' primer complementary to a region (codons 81–86) shared by all $V\alpha 11$ exons (5'-ATGGATCCGAAGTAAGTGCTGAGTC-3')^{17,23,28}. This permitted a search for sequences that might contain the misincorporations located within the codon 8–77 region of the mutated $V\alpha 11$ genes (Fig. 2). The products of this amplification were cloned and 320 randomly chosen, positive bacterial colonies were screened by differential hybridizations with DNA oligomers complementary to mutant (23TAC, 39TAC; Fig. 2) $V\alpha 11.1$ sequences²⁹. Twenty-eight colonies that preferentially bound the labelled oligomers were identified and sequenced. However, none contained any mutant nucleotide(s); 20 inserts contained the germ-line $V\alpha 11.1$ sequence, 3 were $V\alpha 11.2$ and 1 represented

the $V\alpha 11.3$ gene segment. Four examples of a novel $V\alpha 11.3$ -like sequence were observed that may represent a fourth $V\alpha 11$ exon present in B10.A mice (not shown).

The consequences of $V\alpha$ mutation in GC T cells are unknown. Mutations altering the specificity of thymus-selected TCR could confer pathological autoreactivity; this may be averted by the selection implicit in the divergent P:nP ratios for germ-line and mutant $V\alpha 11$ rearrangements. Additionally, GC T cells may not be long lived (T-cell memory is not generated in GC) nor reach significant frequencies because of their long doubling time^{4,15}. Nonetheless, it may be that some mutant T cells return to the peripheral lymphocyte pool (Fig. 3). Cells bearing mutations at codons 90 and -3, consistent with the antigen-driven circulation of T lymphocytes^{15,16}. The GC microenvironment is an important site for the mutational diversification and selection of B cells; interestingly, it may function similarly for T lymphocytes.

Note added in proof: Recently Q. Zhu in our laboratory has accomplished PCR amplification of $V\alpha 11/J\alpha 84$ rearrangements from single $V\alpha 11^+$ T cells dissected from GCs. Four VJ products have been sequenced; all represent in-frame rearrangements and one contains a single misincorporation at codon 49, yielding an average mutation frequency of 9.9×10^{-4} bp⁻¹. □

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Decreased sensitivity to tumour-necrosis factor but normal T-cell development in TNF receptor-2-deficient mice

Sharon L. Erickson, Frederic J. de Sauvage, Kristine Kikly, Karen Carver-Moore, Sharon Pitts-Meek, Nancy Gillett, Kathleen C. F. Sheehan*, Robert D. Schreiber*, David V. Goeddel & Mark W. Moore†

Departments of Cell Genetics, Molecular Biology, Endocrine Research and Pharmacology, Genentech, 460 Point San Bruno Boulevard, South San Francisco, California 94080, USA

* Center for Immunology, Department of Pathology, Washington University School of Medicine, St Louis, Missouri 63110, USA

TUMOUR necrosis factor (TNF) elicits multiple biological effects through two distinct cell surface receptors, TNF-R1 (p55) and TNF-R2 (p75). Most TNF-mediated biological responses, such as cell death, gene induction, antiviral activity and cytokine production, have been attributed to TNF-R1 (refs 1–5). Gene targeting of this receptor confirms its role in the lethality attributable to low doses of lipopolysaccharide after sensitization with D-galactosamine^{6,7}; surprisingly, the toxicity of high doses of lipopolysaccharide was unaffected. The function of TNF-R2 is less well understood, although there are data supporting a role in T-cell development and the proliferation of cytotoxic T lymphocytes^{8,9}. To clarify the physiological role of TNF-R2, we have generated mice deficient in this receptor by gene targeting. The TNF-R2^{-/-} mice show normal T-cell development and activity, but we find that they have increased resistance to TNF-induced death. Additionally, such mice injected subcutaneously with TNF show a dramatic decrease in tissue necrosis, indicating that this receptor plays a role in the necrotic effects of TNF.

To investigate the functions of TNF, we generated mice that do not express TNF-R2. A targeting vector containing a neomycin-resistance (*neo'*) cassette inserted into the second exon of a 9-kilobase (kb) TNF-R2 genomic clone (Fig. 1a) was electroporated into embryonic stem (ES) cells. Gene targeting events were detected at a frequency of ~1 per 60 ES colonies, and three colonies were selected for microinjection into blastocysts. All three gave germline transmission and were interbred to generate homozygous gene targeted mice (TNF-R2^{-/-}) (Fig. 1b). These mice were viable and showed no overt phenotypic abnormalities.

To confirm that the TNF-R2 gene was correctly targeted, mice were challenged with lipopolysaccharide (LPS) which results in shedding of the TNF-R2 extracellular domain (sTNF-R2)¹⁰. We detected soluble TNF-R2 in serum from wild-type mice (TNF-R2^{+/+}) but not in serum from TNF-R2^{-/-} mice, confirming that the gene is no longer functional (Fig. 1c). The levels of TNF present in the serum following LPS challenge were significantly increased in TNF-R2^{-/-} mice compared with wild-type mice. This may be due to the absence of sTNF-R2 in the serum, where it could contribute to the removal of TNF, to masking of the TNF antigen, or to the lack of TNF-R2 on cells, leaving only TNF-R1 to bind and remove circulating TNF. The TNF increase subsides after seven hours, as in control mice (Fig. 1d).

It has been shown that TNF is a major mediator of damage during sepsis and that TNF receptor-IgG chimaeras or antibodies against TNF can protect against LPS-induced lethality^{11–13}. The TNF-R2^{-/-} mice are not more sensitive to high doses of

FIG. 1 Targeting of the TNF-R2 gene by homologous recombination. a, Structure of the human TNF-R2 gene and the gene targeting vector. b, Genotyping by Southern analysis of mouse genomic DNA. Genotypes of mice are indicated as wild-type (+/+), heterozygous (+/-), and homozygous-receptor ablated (-/-). c and d, Mice were injected with 300 µg LPS and bled 90 min (grey bars) and 7 h later (black bars). Levels of soluble sTNF-R2(c) and TNF(d) were analysed by ELISA (K.C.F.S., K. K. Pincard, C. Arthur, D.V.G. and R.D.S., manuscript in preparation).

METHODS. A genomic clone was isolated from a mouse 129 library. A 9-kb subclone was used to construct the targeting vector. A neomycin-resistance gene under control of the P_{gk} promoter²⁰ was inserted into the *Bst*BI site in the second exon, which contains the signal peptide region of TNF-R2, using synthetic DNA linkers. An oligonucleotide probe was designed from sequences 3' to those present on the targeting vector. After homologous recombination, the mutated allele was detected by the addition of an *Eco*RI site from the *neo'* gene. The targeting vector (20 µg) was linearized and electroporated at 275 V, 200 µF using a BTX 300 electroporator into ES D3 C12, a subclone of D3 ES cells²¹. Cells were selected for G418 at 300 µg ml⁻¹ for 10 d. Single colony wells were expanded for DNA isolation and Southern blot analysis. The targeting frequency was 15 of 872 clones screened. Three clones were selected to generate chimaeric mice by microinjection of the targeted ES cell into the blastocoel cavity of 3.5-d C57BL/6J blastocysts²². Chimaeric males were mated with C57BL/6J females and agouti-colour offspring screened for germline transmission by PCR analysis for the *neo'* gene, confirmed by Southern blot analysis of tail DNA. Heterozygous mice were interbred to obtain homozygous animals. Genomic DNA was isolated from tail snips of the offspring, digested with *Eco*RI and analysed by Southern blot hybridization with the oligonucleotide probe shown in a.

LPS (Table 1), despite the transient increase in TNF levels 90 min after LPS injection (Fig. 1d). Instead, there is a trend of decreased sensitivity to LPS in the mid-range dosage. D-galactosamine treatment enhances LPS sensitivity owing to its toxic effects upon hepatocytes. Unlike TNF-R1-deficient mice, which had much greater tolerance to LPS and D-galactosamine⁶, there is no significant difference in sensitivity in this model between wild-type and TNF-R2^{-/-} mice (Table 1). However, TNF-R2^{-/-} mice are much less sensitive to TNF: 10 of 11 TNF-R2^{-/-} mice survived injection of 10 µg mTNF, whereas all wild-type mice died at this dose (Table 1). Although TNF-R2^{-/-} mice are less sensitive than wild-type mice, they are not completely resistant, as they died when higher doses were given (Table 1). These results demonstrate that TNF-R2 contributes to TNF toxicity as it appears to lower the lethal dose. Previous work has shown that TNF-R1 is the dominant receptor involved in TNF toxicity^{2,3,8,14}. Our work supports this conclusion and expands it to include an enhancing role for TNF-R2.

Injection of murine TNF under the skin of mice causes a rapid necrosis of the surrounding tissues (K.C.F.S. and R.D.S., manuscript in preparation). As most of the cytotoxic effects of TNF have been attributed to TNF-R1, it was unexpected to find that the TNF-R2^{-/-} mice showed markedly reduced tissue necrosis in response to TNF (Fig. 2a). This difference in necrosis between the TNF-R2^{-/-} and wild-type mice is seen upon visual inspection of the injected area. Histological analysis (Fig. 2b) reveals that wild-type mice have an area of ulceration that extends deep into the dermis, whereas TNF-R2^{-/-} mice are only mildly affected; in both cases, the cellular infiltrate is composed of lymphocytes and neutrophils.

T- and B-cell populations from unchallenged mice were analysed for cell surface markers by cytofluorometric analysis; no significant differences in cell numbers or composition of lymphocyte subsets were found. Functional analysis of T cells by mixed lymphocyte response, alloctotoxic T lymphocyte (CTL) response, protein-antigen-specific responses and stimulation by concanavalin A did not reveal any changes in responsiveness. Similarly, B cells were still responsive to LPS (data not shown). As TNF-R2 participates in thymocyte proliferation, we analysed

† To whom correspondence should be addressed.

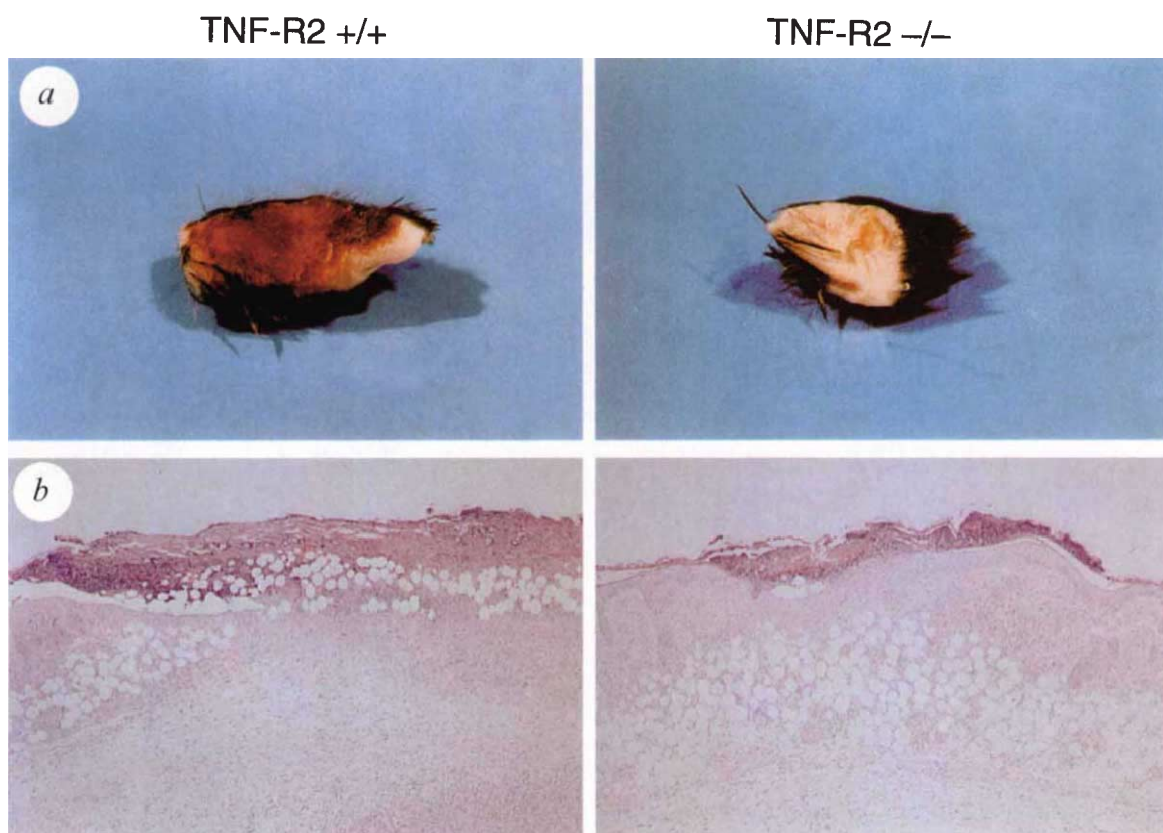
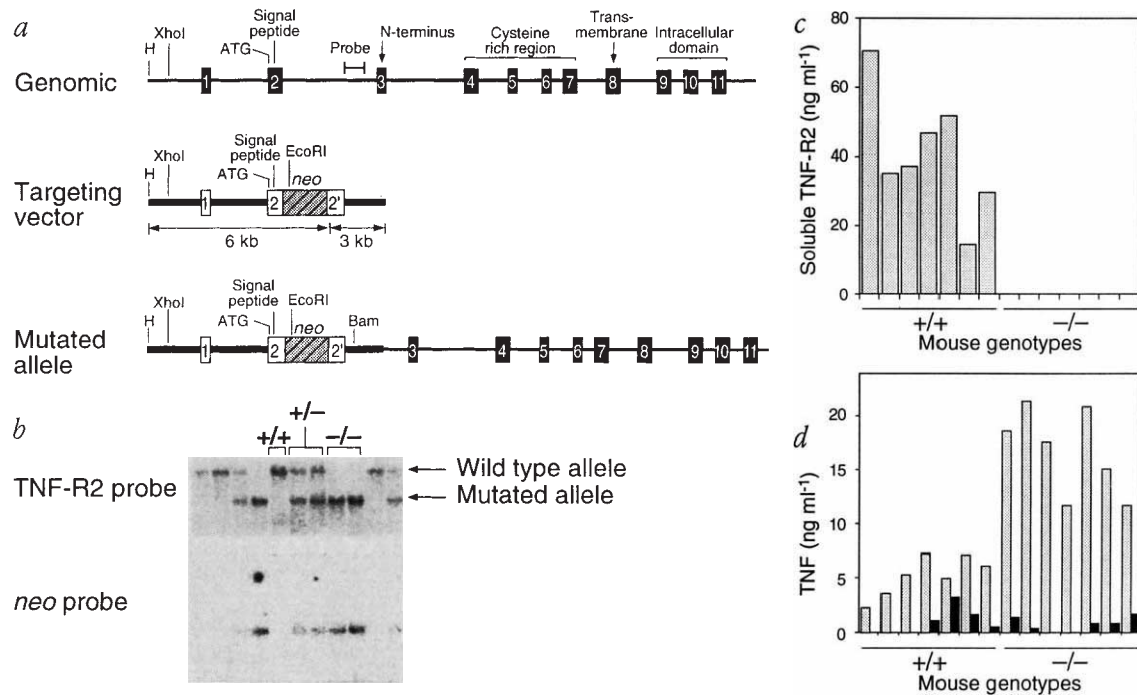


FIG. 2 Tissue necrosis in response to TNF injections. Both wild-type and TNF-R2^{-/-} mice were injected subcutaneously with 3 µg murine TNF for 5 d. Animals were killed for histology preparations. **a**, Skin patches

from the site of injection. **b**, Histological sections at 154× magnification of tissues stained with haematoxylin and eosin. Three mice per group were tested and a representative of each is shown.

thymocytes for cell surface markers of differentiation, including CD4, CD8, heat-stable antigen, $\alpha\beta$ T-cell receptor (TCR), and $\gamma\delta$ TCR. We did not discover any significant alteration in these indicators of T-cell development in TNF-R2^{-/-} mice (Fig. 3) as the ratio of CD4⁺ and CD8⁺ single positive cells to CD4⁺CD8⁺ immature thymocytes was unaltered. Additionally, the amount of $\alpha\beta$ TCR on the cell surface was unaltered. To investigate the role of this receptor in thymic T-cell deletion and development, we backcrossed TNF-R2^{-/-} mice onto BALB/c mice, which normally have an intrathymic deletion of TCR V β 5 chains due to mls, or superantigen, reactivity, but V β 8 chains are not deleted^{15,16}. We found no alteration of V β selection in TNF-R2^{-/-} mice (Fig. 4). Although TNF can induce thymocyte proliferation *in vitro* via activation of TNF-R2 (ref. 9), our results demonstrate that signalling through TNF-R2 is not necessary for T-cell development in TNF-R2^{-/-} mice. There could be

changes to compensate for the loss of TNF-R2, or TNF-induced proliferation of thymocytes may not be physiologically relevant. Another receptor, lymphotoxin- β R (LT- β R), which is specific for the LT α -LT β heterotrimer¹⁷, may have an important immunological function, and targeted ablation of LT- α disrupts peripheral lymphoid development¹⁸. As neither the TNF-R1-nor the TNF-R2-targeted mutation gives a similar phenotype, LT- β R is probably the receptor involved in LT α -related peripheral lymphoid development.

Some defects in the immune response of TNF-R1-deficient mice were apparent, as they had an increased susceptibility to infections by *Listeria monocytogenes*, with increased colony counts in spleen and liver after 3 days and increased mortality over 6 days^{6,7}. Challenge with a sublethal dose of *L. monocytogenes* did not reveal any significant differences between wild-type and TNF-R2^{-/-} mice after 3 days, but mortality was increased over 6 days in TNF-R2^{-/-} mice, with 9/28 mice dying compared to 1/26 for wild-type with an inoculum of 1–3 $\times 10^5$ bacterium. This overall sensitivity of TNF-R2^{-/-} mice to *Listeria* infection appears to be less severe than in TNF-R1-deficient mice as most of those animals died from a 5-fold-lower inoculum^{6,7}, but a direct comparison of the two types of receptor-deficient mice is needed.

Although TNF-R2 can signal thymocyte proliferation⁸, our data indicate that this role is not vital and that T cells develop normally in TNF-R2^{-/-} mice. Our results show that this receptor is not responsible for the toxic effects of LPS or TNF but that it increases sensitivity to TNF. The alteration in tissue necrosis

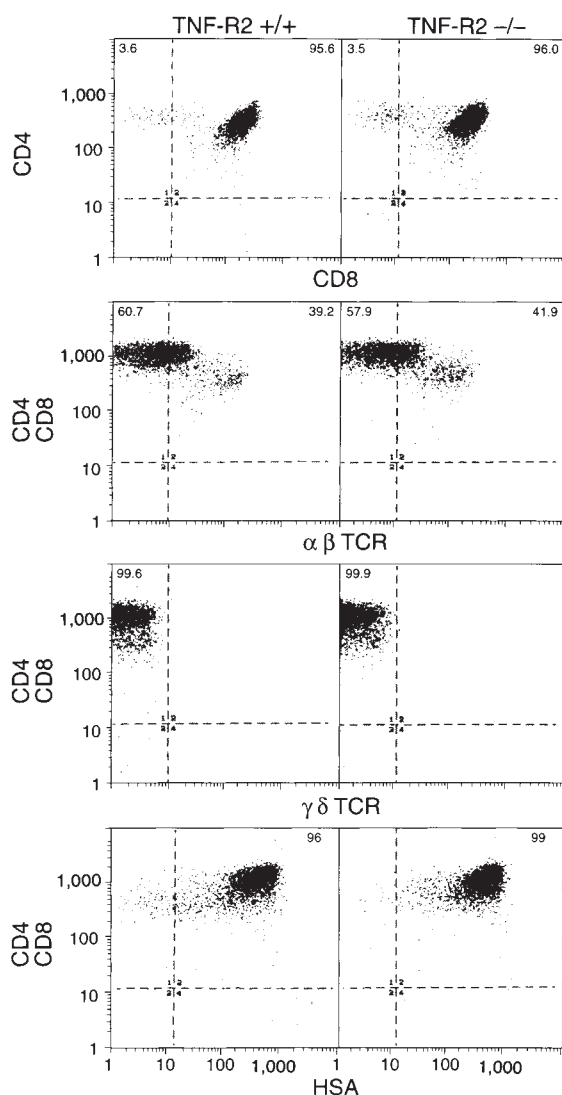


FIG. 3 Thymocyte analysis in TNF-R2^{-/-} mice. The expression patterns of the indicated cell surface markers were analysed in TNF-R2^{+/+} and -/- mice. Single-cell suspensions were prepared from the thymus of 6-week-old mice and stained with FITC-conjugated antibodies specific for either CD8, or CD8 and CD4 (Becton-Dickinson) and phycoerythrin-conjugated anti-CD4 (Becton-Dickinson), $\alpha\beta$ TCR (Boehringer-Mannheim), $\gamma\delta$ TCR (Boehringer-Mannheim), or heat stable antigen (Pharmingen). Cells were fixed in 1% paraformaldehyde before analysis on a FACScan (Becton-Dickinson).

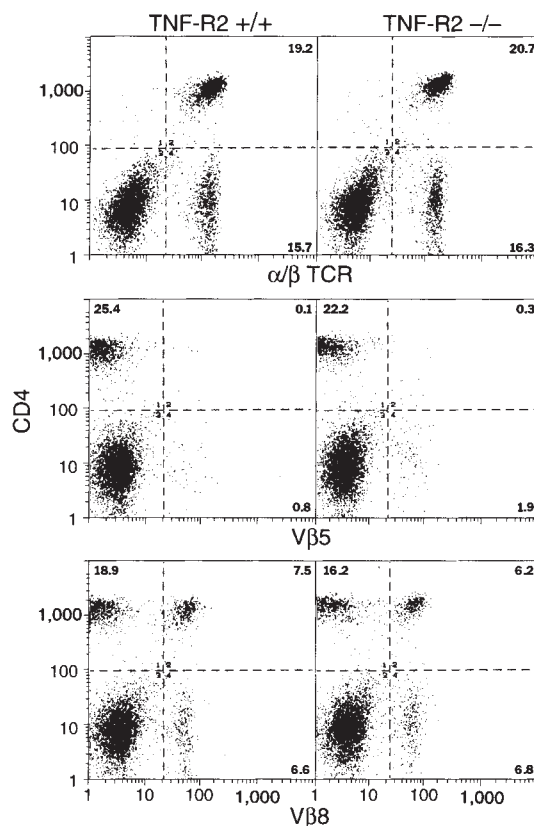


FIG. 4 T-cell receptor selection in TNF-R2^{-/-} mice. Thymocytes were analysed for the expression of V β 5 (which should be deleted on the BALB/c background) and V β 8 (which should undergo positive selection). METHODS. Single-cell suspensions were prepared from the thymus of 6-week-old mice and stained with FITC-conjugated antibodies (Pharmingen) specific for either V β 5 or V β 8 and a phycoerythrin-conjugated anti-CD4 (Becton-Dickinson). Cells were fixed in 1% paraformaldehyde before analysis on a FACScan (Becton-Dickinson).

TABLE 1 Effects of LPS and TNF- α on TNF-R2 $^{-/-}$ mice

LPS (μ g)	Dose		Lethality	
	D-galactosamine (mg)*	TNF- α † (μ g)	TNF-R2 $^{+/+}$	TNF-R2 $^{-/-}$
800	—	—	5/5	5/5
600	—	—	9/10	4/10
500	—	—	5/5	2/5
300	—	—	3/4	1/4
100	—	—	0/4	0/4
100	20	—	5/5	5/5
10	20	—	5/5	5/5
1.0	20	—	5/5	5/5
0.1	20	—	9/10	9/10
0.01	20	—	6/7	6/7
0.001	20	—	1/5	2/5
		10	10/10	1/11
		15	4/4	4/4

* Mice were injected intraperitoneally with the indicated amount of LPS (*Salmonella abortus equi*; Sigma) with or without D-Galactosamine (20 mg per mouse) in 0.2 ml Hank's balanced salt solution. Lethality was monitored over 3 days and indicated as lethality/total injected. P values were <0.006 for LPS toxicity and <0.01 for the TNF study.

† Recombinant murine TNF- α (10 μ g; Genentech) was injected intravenously.

following TNF injection also demonstrates the decreased sensitivity to TNF in TNF-R2 $^{-/-}$ mice. This decreased sensitivity is consistent with the 'ligand passing' model in which TNF-R2 helps recruit TNF for interaction with TNF-R1 and lowers the concentration of TNF needed for TNF-R1 signal transduction¹⁹. Alternatively, TNF-R2 could synergize with TNF-R1 in transducing the TNF signal, or TNF-R2 could perform other

functions in this system that cause additional damage. Neither TNF-R1- nor TNF-R2-deficient mice are completely insensitive to high doses of LPS alone, but both show decreased sensitivity to TNF. Thus, either both receptors contribute to LPS-induced septic shock or other cytokines produced during sepsis act independently of TNF and contribute greatly to toxicity. Interbreeding of TNF-R1 $^{-/-}$ and TNF-R2 $^{-/-}$ mice to generate double-receptor-ablated mice should help clarify this issue. Strategies for therapeutic intervention of sepsis may be influenced by these results. □

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Activation of the kinase Pelle by Tube in the dorsoventral signal transduction pathway of *Drosophila* embryo

Jörg Großhans, Andreas Bergmann, Pascal Haffter & Christiane Nüsslein-Volhard

Max-Planck-Institut für Entwicklungsbiologie, Abteilung III (Genetik), Spemannstraße 35, 72076 Tübingen, Germany

THE concentration of Dorsal protein in the nucleus determines cell fate along the dorsoventral axis of the *Drosophila* embryo^{1–3}. The *dorsal*-group genes and the *cactus* gene are required for production and transmission of a localized signal on the ventral side of the embryo^{4,5} which determines the position of the highest nuclear concentration of Dorsal protein^{1–3}. The ventralizing signal produced in somatic cells⁶ is transmitted through the perivitelline space⁷ to the integral membrane protein Toll⁸. Inside the embryo it leads to dissociation of the cytoplasmic Dorsal–Cactus complex and subsequent nuclear localization of Dorsal protein^{9,10}. Two components are known to mediate the signal transduction between Toll and Dorsal–Cactus^{11,12}: Pelle, a serine/threonine protein kinase¹³, and Tube, a protein with an unknown biochemical activity¹⁴. Here we construct gain-of-function alleles of *pelle* and *tube* and show that *pelle* functions downstream of *tube*. In addition, Pelle and Tube interact directly with one another. We propose that Tube is a direct activator of the protein kinase Pelle.

In dorsolateralized embryos, Dorsal protein is restricted to the cytoplasm, whereas in ventralized embryos all nuclei around the egg

circumference contain Dorsal protein^{1–3}. Analysis of double mutants combining a ventralizing allele of *Toll* and a dorsalizing allele of the *dorsal*-group genes established their functional order. It has been shown that only *pelle*, *tube* and *dorsal* function downstream of *Toll*, and the remaining *dorsal*-group genes (such as *snake* and *nudel*) function upstream^{4,12}. But the functional order of *pelle* and *tube* was unclear as no ventralizing alleles for these genes were available. We constructed ventralizing gain-of-function alleles of *pelle* and *tube* by replacing the intracellular kinase domain of a gain-of-function allele of the receptor tyrosine kinase Torso, *torso*⁴⁰²¹ (refs 15, 16), by the *pelle* or *tube* coding sequences. We refer to these constructs as *tor*⁴⁰²¹*pelle* and *tor*⁴⁰²¹*tube*. A similar strategy has been used to obtain an activated form of *D-raf* in the investigation of the *sevenless* signal transduction pathway¹⁷. RNA encoding the fusion proteins was synthesized *in vitro* and microinjected into embryos from females of various genotypes.

Injection of *tor*⁴⁰²¹*pelle* RNA into embryos from *pelle* females, and *tor*⁴⁰²¹*tube* RNA into embryos from *tube* females induced dorsolateral and ventrolateral pattern elements. A similar effect was observed when *tor*⁴⁰²¹*pelle* or *tor*⁴⁰²¹*tube* RNA was injected into embryos from females mutant for the upstream *dorsal*-group gene *snake*. Near the injection site, embryos developed ventral denticle belts and filzkörper (Table 1 and Fig. 1a, c, d) which are absent in mutant embryos (Fig. 1b). In addition, expression of *twist* (Fig. 1e) was induced at the injection site, indicating the highest levels of nuclear Dorsal protein¹. To confirm the gain-of-function effect, we analysed the position of the posterior midgut invagination, which normally occurs on the dorsal side. When RNA was deposited on the dorsal side of the egg, we observed a posterior midgut invagination at a ventral position, opposite the site of RNA deposition (Fig. 1f–h), indicating a clear reversal of dorsoventral polarity. There is no strict requirement for the *torso*⁴⁰²¹ allele, as fusion constructs with

FIG. 1 Induction of dorsoventral pattern elements by *tor*⁴⁰²¹*pelle* RNA. **a–d**, Cuticle preparations of embryos from wild-type (**a**), *tube* (**b, d**) and *snake* (**c**) females. **a** and **b**, Not injected; **c** and **d**, injected with *tor*⁴⁰²¹*pelle* RNA. Filzkörper (fk) and ventral denticle belts (vd) are indicated. These structures are absent in completely dorsalized embryos (**b**). Fusion to *torso* is necessary for activation, as unmodified *pelle* and *tube* RNA had no effect when injected into embryos from *snake* females (data not shown). Similarly, *torso*⁴⁰²¹ RNA injected into embryos from *snake* females did not induce filzkörper (data not shown). **e**, An embryo from a *tube* female injected with *tor*⁴⁰²¹*pelle*3' RNA (see below) and stained for Twist (antibody provided by R. Reuter). **f–h**, Living embryos from *snake* females, uninjected (**g**) or injected with *tor*⁴⁰²¹*pelle* RNA either dorsally (**h**) or ventrally (**f**). The position of posterior midgut invagination is indicated by arrows.

METHODS. The *tor*⁴⁰²¹*pelle* fusion construct with amino-acid residues 1–455 of Torso⁴⁰²¹ (refs 16, 26) and 2–501 of Pelle¹³ contains the signal peptide, the extracellular domain, and the transmembrane peptide of Torso, with Pelle as its intracellular domain. In the *torso*⁴⁰²¹ allele, a single amino-acid residue is changed in the extracellular domain²⁶. DNA encoding Pelle, with *Bst*III and *Xho*I sites, was prepared by polymerase chain reaction (PCR) and cloned into the vector pB4021 (ref. 26) missing a *Bst*III–*Sal*I fragment. The related construct *tor*⁴⁰²¹*pelle*3', which contains the 3' untranslated region of the *pelle* cDNA in addition to the coding sequence, was constructed similarly. RNA was synthesized *in vitro* and injected as described²⁶ at about 1 mg ml⁻¹ into the dorsal or ventral cortical zone in the posterior half of embryos, at about 30% egg length, before pole cell formation. The allelic combinations *tub*^{R5.6}/*Df*(3R)*hkb*^{XM3} and *snk*⁰⁷³/*snk*²²⁹ were used.

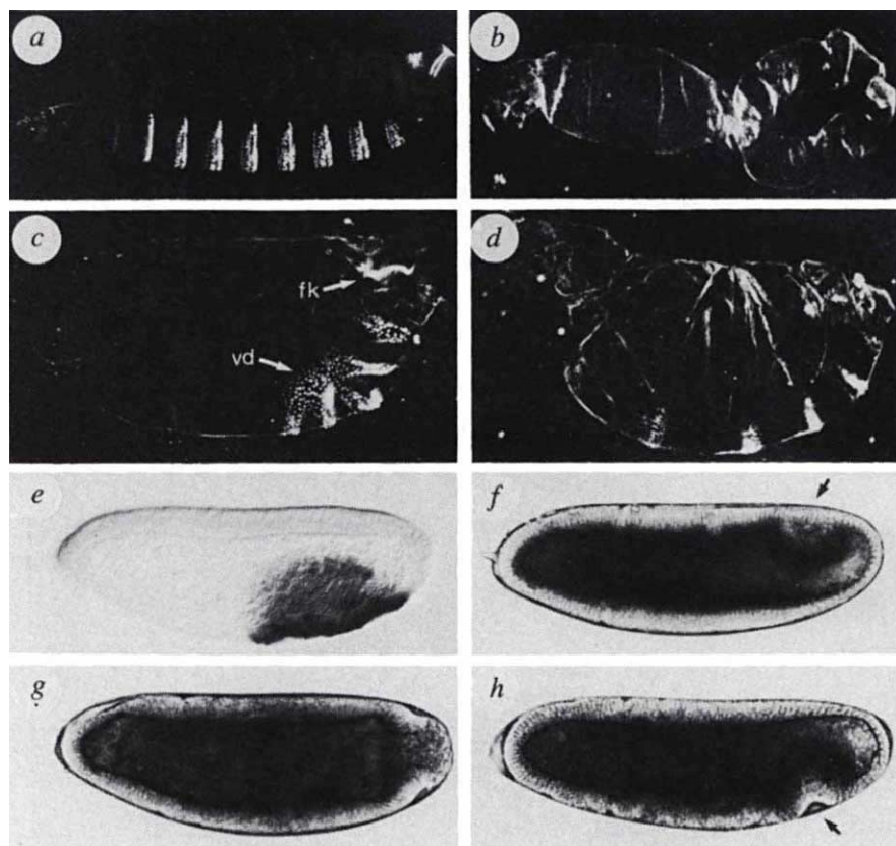


FIG. 2 Pelle and Tube interact physically. **a**, Interaction in the two-hybrid system. Yeast strains containing a *LEU2* and a *lacZ* reporter gene, and plasmids encoding the indicated combination of *lexA* and activation-tagged fusion proteins, were streaked out on synthetic medium without leucine and incubated for four days. The assay for *lacZ* reporter gene activation gave a similar result (data not shown). The slow growth of some yeast strains containing the *lexA*–*pelle* fusion protein is due to the property of *LexA*–*pelle* to activate the reporter gene weakly by itself, as seen in the combination *lexA*–*pelle*/JG4-5. **b**, Association *in vitro*. Indicated combinations of radiolabelled and unlabelled protein were subjected to immunoprecipitation with an antibody specific for the unlabelled protein, and analysed by SDS–PAGE (upper panel, immunoprecipitates (IP), lower panel, supernatants (SN)). **c**, Pelle phosphorylates Tube *in vitro*. Pelle immunoprecipitates prepared from wild-type (OR) and *pelle* embryonic extracts (rm8, 019) were assayed for their ability to phosphorylate Tube *in vitro* (upper panel). Presence of Pelle was assayed by western blotting of total embryonic extracts (lower panel). **d**, Phosphorylation of bacterially expressed Tube, Cactus and Dorsal protein by Pelle immunoprecipitates prepared from *nudel* (*ndl*) and *Toll* (*TI*^{10b}) embryonic extracts, followed by SDS–PAGE (upper panel, short exposure; middle panel, long exposure of the same gel). Pelle immunoprecipitates were omitted in the buffer controls. Lower panel, autophosphorylation of Pelle immunoprecipitates.

METHODS. DNA containing the coding sequences of the indicated genes^{8,13,14,19,23,27,28} (except *toll*^{10b} with codons 830–1097) and appropriate ends for cloning was prepared by PCR and cloned into the vectors pEG202 and pJG4-5 in the reading frames of *lexA* and of the activation tag, respectively²⁹. Yeast strains contained a chromosomal *LEU2* reporter gene (EGY48) (ref. 29) and the *lacZ* reporter gene pSH18–34

(constructed by S. Hanes). Assays for reporter gene activation were done on synthetic galactose/raffinose medium without leucine. Cloning, expression in *Escherichia coli*, and purification of 6 × His-tagged Tube¹⁴, Pelle¹³, Cactus and Dorsal²³ proteins were done with the vectors pQE12 and pQE30 (Qiagen). Production and affinity purification of Tube-, Pelle-, Cactus- and Dorsal-specific antibodies and western blot analysis were modified from refs 1 and 10. Reticulocyte lysate (5 μl) (Amersham) containing ³⁵S-labelled protein was incubated with 100 ng bacterially expressed, purified protein (6 × His-tagged Tube, Cactus, Dorsal proteins; bacterial control extract pQE12) for 30 min at 25 °C. Immunoprecipitation was done with 2 μg of an antibody specific for the unlabelled protein, pre-bound to protein A–Sepharose beads (Pharmacia) in immunoprecipitation (IP) buffer (50 mM HEPES/NaOH, pH 7.5, 150 mM NaCl, 10% glycerol, 0.5% Triton X-100, with protease and phosphatase inhibitors) for 2 h at 4 °C followed by five washing steps with buffer, and SDS–PAGE. Protein in the supernatants was precipitated with trichloroacetic acid and subjected to SDS–PAGE. Embryonic extracts were obtained by lysing 0–4-h embryos of the indicated maternal genotypes (*plf*^{rm8}/*Df*(3R)D605, *plf*⁰¹⁹/*Df*(3R)D605, *ndl*⁰⁴⁶/*ndl*⁰⁹³, *TI*^{10b}/+) in IP buffer. In protein kinase assays, Pelle immunoprecipitates were incubated with 100 ng bacterially expressed, purified 6 × His-tagged Tube, Cactus or Dorsal protein in 30 μl kinase buffer³⁰ for 30 min at 25 °C. After centrifugation, Pelle immunocomplexes were washed four times with IP buffer and used for autophosphorylation analysis. Supernatants were immunoprecipitated with either Tube-, Cactus- or Dorsal-specific antibodies. Analysis was by SDS–PAGE and fluorography. The gel in **d** was exposed overnight without an intensifying screen (upper and lower panels) or for three days with an intensifying screen (middle).

the corresponding part of wild-type *torso* show similar effects, although their activity is weaker (data not shown). Thus, fusions of *pelle* and *tube* with *torso* bypass the normal requirement for upstream *dorsal*-group genes for the induction of dorsoventral pattern elements and are able to induce polarity independently of the prelocalized signal.

The ventralizing constructs, *tor*⁴⁰²¹*pelle* and *tor*⁴⁰²¹*tube*, were used to determine the relative position of the two gene functions in the signalling pathway. Injection of *tor*⁴⁰²¹*pelle* RNA into

embryos from *tube* females resulted in the same rescue response as injection into embryos from *snake* or *pelle* females (Fig. 1c, d and Table 1). In contrast, we observed no effect of injection of *tor*⁴⁰²¹*tube* RNA into embryos from *pelle* females (Table 1). Neither of the fusion constructs was able to induce dorsoventral pattern elements in embryos from *dorsal* females (Table 1), consistent with previous findings that *dorsal* is the final target of the dorsoventral signal transduction pathway^{4,11}. As disruption of *pelle* activity blocks signalling by *tor*⁴⁰²¹*tube*, whereas on the

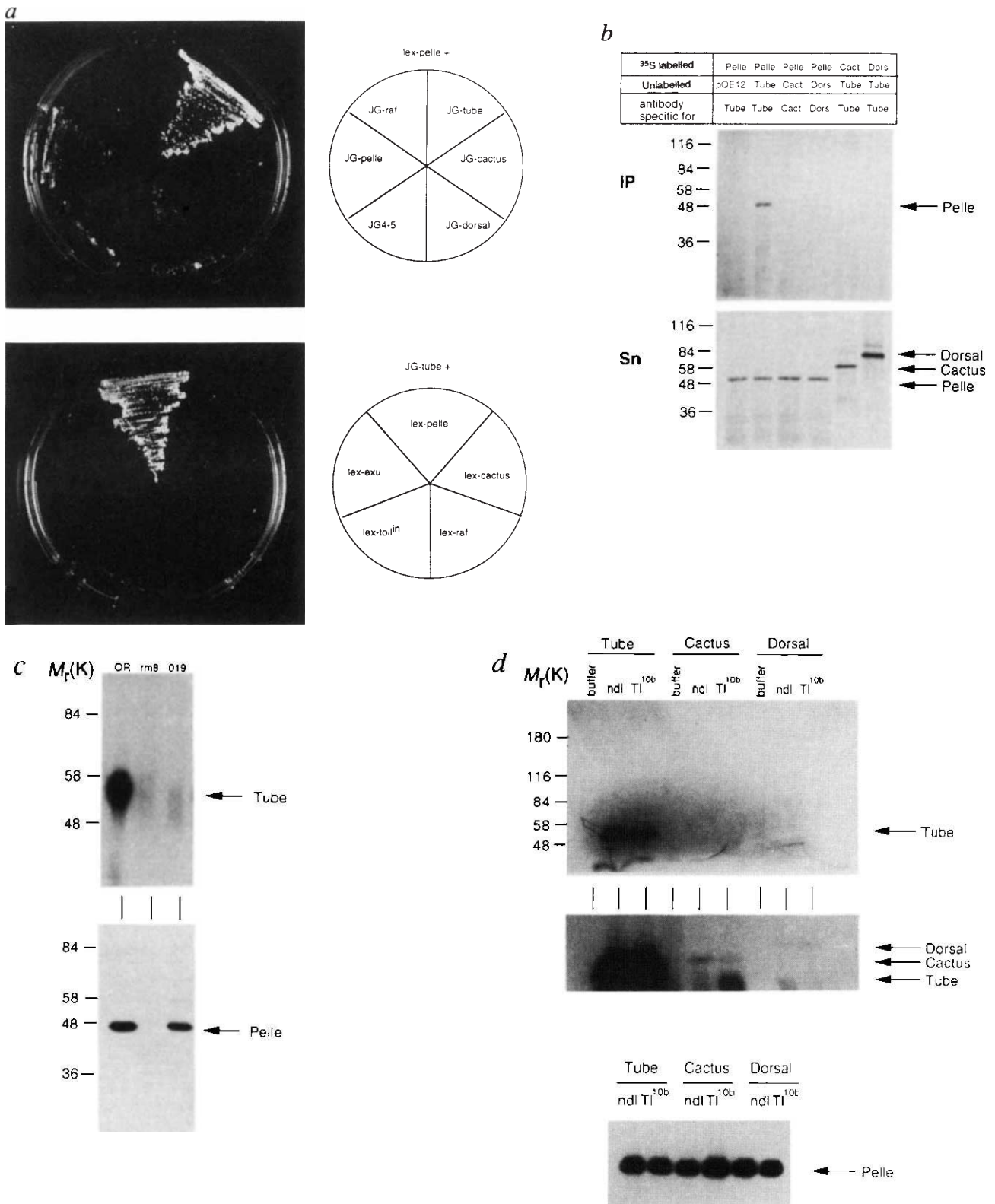


TABLE 1 *tor*⁴⁰²¹*pelle* RNA and *tor*⁴⁰²¹*tube* RNA injections

RNA injected (1 mg ml ⁻¹)	Maternal genotype	Number of differentiated embryos	Cuticle phenotype				Partial rescue (FK or VD)
			DO	FK	FK + VD	VD	
<i>tor</i> ⁴⁰²¹ <i>pelle</i>	<i>snake</i>	114	41	46	22	5	64%
	<i>tube</i>	77	31	32	14	0	62%
	<i>pelle</i>	42	14	6	15	7	66%
	<i>dorsal</i>	108	105	(3)	0	0	0%
<i>tor</i> ⁴⁰²¹ <i>tube</i>	<i>snake</i>	111	18	15	29	49	84%
	<i>tube</i>	48	5	3	11	29	90%
	<i>pelle</i>	36	36	0	0	0	0%
	<i>dorsal</i>	29	29	0	0	0	0%

*tor*⁴⁰²¹*pelle* and *tor*⁴⁰²¹*tube* RNA (about 1 mg ml⁻¹) were injected into embryos from females of the indicated genotypes before pole-cell formation. RNA was deposited either in the dorsal or ventral cortical zone at about 30% egg length. There was no difference in frequency of rescue between embryos injected dorsally and ventrally. Cuticle preparations of differentiated embryos were scored according to the following categories: completely dorsalized (DO), complete filzkörper or filzkörper material present (FK), complete or partial ventral denticle belts present (VD), and filzkörper or filzkörper material and ventral denticle belts present (FK + VD). Allelic combinations used: *snk*⁰⁷³/*snk*²²⁹, *tub*^{R5.6}/*Df*(3R)hkb^{XM3}, *plf*^{rm8}/*plf*⁰⁷⁸, *plf*^{rm8}/*Df*(3R)D605, *dl*¹/*Df*(2L)TW119, *dl*^H/*Df*(2L)TW119. Preparation and analysis of cuticles was as described²⁵. Construction of *tor*⁴⁰²¹*tube*, encoding amino-acid residues 2–462 of Tube¹⁴, was similar to *tor*⁴⁰²¹*pelle* construction (Fig. 1 legend).

other hand *tube* is not required for function of *tor*⁴⁰²¹*pelle*, we conclude that *tube* acts upstream of *pelle* in the signalling cascade.

Certain allelic combinations of *pelle* and *tube* show dominant genetic interactions that suggest a molecular interaction¹². We used the two-hybrid system in yeast¹⁸ to test for a direct interaction. Pelle and Tube interact specifically, as the reporter gene was activated above basal levels (40 times higher, using the lacZ reporter gene) only in yeast strains that contained Tube and Pelle fusion proteins (Fig. 2a). The combinations Pelle–Dorsal and Pelle–Cactus did not activate the reporter gene, although the functional integrity of Dorsal and Cactus constructs could be shown by confirming their well established interaction^{9,19,21} by the two-hybrid system (data not shown). In a preliminary screen for proteins interacting with Pelle, we isolated a *tube* complementary DNA, confirming the specificity of the Tube–Pelle interaction (unpublished observations).

To demonstrate the direct interaction of Tube and Pelle by independent means, we reconstituted their association *in vitro*. Bacterially expressed unlabelled protein was added to radiolabelled protein in reticulocyte lysate and subjected to immunoprecipitation with an antibody specific for the unlabelled protein. Of all the combinations tested, coimmunoprecipitation of Pelle was only detected when Tube and Pelle were present in the mixture (Fig. 2b, upper panel). Analysis of the supernatants showed that only a small proportion of Pelle was coimmunoprecipitated, indicating weak binding of the two proteins (Fig. 2b, lower panel).

We also observed that Tube is phosphorylated by Pelle immunoprecipitates *in vitro*. Pelle was enriched from embryonic extracts by immunoprecipitation with a Pelle-specific antibody, and incubated with bacterially expressed Tube under phosphorylation conditions. Phosphorylation of Tube is due to Pelle, because it is found with immunoprecipitates from wild-type embryos, but not with immunoprecipitates from certain *pelle* alleles (Fig. 2c, upper panel), which either do not contain detectable amounts of Pelle (*plf*^{rm8}), or contain a mutant form of Pelle (*plf*⁰¹⁹; Fig. 2c, lower panel). The phosphorylation activity is specific as no significant phosphorylation of Cactus or Dorsal protein was found under similar conditions (Fig. 2d, upper panel). But as faint signals of Cactus and Dorsal protein phosphorylation were visible after long exposure (Fig. 2d, middle panel), this experiment *in vitro* does not rule out the possibility that Cactus or Dorsal protein are direct targets of activated Pelle in the embryo¹³. Because the kinase activity in our preparation does not depend on the ventralizing signal (compare *nld* dorsalized with *Tl*^{10b} ventralized extracts; Fig. 2d), and Tube acts upstream of Pelle, the significance of a Pelle-mediated phosphorylation of Tube *in vivo* is not clear. A phosphorylation of Tube in the embryo could be part of a feedback mechanism

regulating signal transduction between Tube and Pelle. Although it might not play a role *in vivo*, we take it as further evidence for a direct interaction of Tube and Pelle.

In summary, our results support a model in which Tube mediates signal transduction between Toll and Pelle by direct interaction with Pelle. The phosphorylation target of activated Pelle is still unknown, but candidates are Cactus, Dorsal protein, or another as-yet unidentified protein. Because the active form of Torso, like other receptor tyrosine kinases, is probably a dimer²², the activating fusion constructs suggest that dimerization may be important for activation of Pelle and Tube. In addition, membrane association may be involved, as fusion with wild-type Torso leads to activation of Pelle and Tube as well, although with lower efficiency. The signalling pathways regulating activation of NF- κ B and Dorsal protein might be conserved, as homologous components are involved: Dorsal protein is a member of the rel/NF- κ B family²³, Cactus is homologous to I- κ B proteins^{19,20} and Toll is partially homologous to the interleukin-1 receptor^{8,24}. Future research will show whether homologues of Pelle and Tube have similar functions in regulation of NF- κ B in vertebrates. □

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A yeast gene necessary for bud-site selection encodes a protein similar to insulin-degrading enzymes

Atsushi Fujita, Chitoshi Oka*, Yukihiko Arikawa†, Tatsuyuki Katagal, Akio Tonouchi‡, Satoru Kuhara‡ & Yoshio Misumi§

National Institute of Bioscience and Human-Technology, Agency of Industrial Science & Technology, 1-1 Higashi, Tsukuba 305, Japan

* Industrial Research Institute of Chiba Prefecture, Kasori, Wakaba-ku, Chiba 264, Japan

† Nagano State Laboratory of Food Technology, Nishibannba, Kurita, Nagano 380, Japan

‡ Graduate School of Genetic Resources Technology, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812, Japan

§ Department of Biochemistry, School of Medicine, Fukuoka University, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-01, Japan

CELLS of the yeast *Saccharomyces cerevisiae* choose bud sites in a non-random spatial pattern that depends on mating type: axial for haploid cells and bipolar for *a/a* diploid cells^{1,2}. We identified a mutant yeast, *axl1*, in which the budding pattern is altered from axial to bipolar. Expression of the *AXL1* gene is repressed in *a/a* diploid cells. With the ectopic expression of *AXL1*, *a/a* cells exhibited an axial budding pattern, thus *AXL1* is a key morphological determinant that distinguishes the budding pattern of haploid cells from that of *a/a* diploid cells². *AXL1* encodes a protein similar in sequence to the human and *Drosophila* insulin-degrading enzymes^{3,4} and to the *Escherichia coli* *ptr* gene product⁵. The axial budding pattern might result from degradation of a target protein by the putative *Axl1* protease.

Two classes of genes are necessary for proper bud-site selection²: *BUD1-BUD2-BUD5* and *BUD3-BUD4*; the former is necessary for both bipolar and axial budding, the latter for converting a bipolar pattern to axial (Fig. 1a). In *a* and *a* cells budding is proposed to be axial, because all *BUD* genes are active (Fig. 1b). In contrast, expression of *BUD3* or *BUD4* may be turned off by the repressor *a1-a2* in *a/a* cells² (Fig. 1b), which may lead to bipolar budding in *a/a* cells. The *axl1-1* mutant was identified when screening for mutants with a bipolar budding pattern (Fig. 2a, b; see legend to Table 1). The position of bud site formation was quantified in cells with a single bud scar and a single bud according to the scheme in Table 1. The *axl1* mutant was mated to *bud3* or *bud4* strains for complementation tests. The *MATa* genes of the two diploids so formed were disrupted to neglect the effect of repressor *a1-a2*. As they exhibited the axial budding pattern, the *AXL1* gene is distinct from *BUD3* and *BUD4*. *AXL1* was cloned by complementation of the bipolar budding phenotype of an *axl1* strain (see Fig. 3 legend). We also observed that the cloned *AXL1* gene did not complement *bud3* and *bud4* strains. Thus, *axl1*, *bud3* and *bud4* belong to different complementation groups. A disruption allele of *AXL1* (*axl1-Δ1::URA3*) was constructed (Fig. 3a). Phenotypes of the *AXL1* null mutation in haploid cells were indistinguishable from those of the *axl1-1* mutant in haploid cells: both budded in a bipolar fashion (Fig. 2c, d), and the *AXL1* null mutation apparently did not affect the bipolar pattern in *a/a* diploid cells (data not shown). Prior studies showed that mutations in genes required for both axial and bipolar budding (*BUD1*, *BUD2* and *BUD5*) are epistatic to mutations of genes required only for axial budding (*BUD3* and *BUD4*)^{2,6,7}. When the epistasis test was done, the *axl1 bud5* strain exhibited the

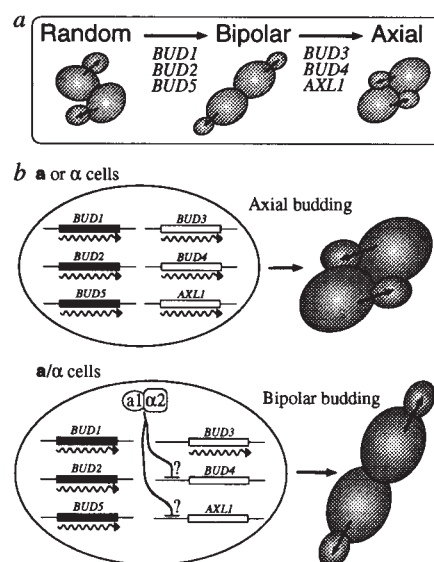


FIG. 1 Models for the morphogenetic pathway for bud-site selection². *a*, Control of budding pattern with *BUD* and *AXL1* genes. The random budding pattern is the basal state. *BUD1*, *BUD2* and *BUD5* are necessary for both bipolar and axial budding. Further addition of *BUD3*, *BUD4* and *AXL1* functions exhibits axial budding. *b*, Two classes of genes control the axial and bipolar budding in *a* and *α* cells and *a/a* cells. Black bars indicate genes required for bipolar and axial budding, white bars only those for axial budding. Wavy arrows under bars indicate that genes are transcribed. It is proposed that *BUD3* or *BUD4* may be repressed by repressor *a1-a2* only found in *a/a* cells². However, transcripts of *BUD3* were detected in all cell types (J. Chant, J. Pringle and I. Herskowitz, personal communication).

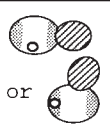
random pattern (Fig. 2e, f) which means that *bud5* is epistatic to *axl1*. Thus, *AXL1* is indeed a class of genes necessary only for axial budding such as *BUD3* and *BUD4*.

The expression of *AXL1* may be controlled by the repressor *a1-a2* in *a/a* diploid cells (Fig. 1b). We examined the pattern of *AXL1* expression in *a* and *α* haploid cells and in *a/a* diploid cells. Figure 2g shows that *AXL1* is transcribed in *a* and *α* haploid cells but not in *a/a* diploid cells. We found that the level of *AXL1* mRNA was higher in *a* cells than in *α* cells. However, quantification of the budding patterns exhibited no significant difference between *a* and *α* cells (Table 1). We have no explanation for this occurrence. *AXL1* is the first known gene that is involved in bud-site selection, the expression of which is cell-type-specific.

We determined the nucleotide sequence of a 5.3 kilobase (kb) DNA segment that complements the *axl1* mutation (Fig. 3a). This region contains a 3,624 base pair (bp) open reading frame (ORF) that potentially encodes a 1,208 amino-acid polypeptide of 138K (Fig. 3a, b). The 5'-region of *AXL1* has sequences that resemble *a1-a2* repression sites (Fig. 3b) to which the *a1-a2* repressor may bind. To examine the effect of ectopic expression of *AXL1*, we prepared a construct (YEUp-*AXL1*Lp) which can express *AXL1* by the promoter of *LEU2* in all cell types (see legend to Table 1). The ectopic expression of *AXL1* permitted *a/a* cells to exhibit axial budding at high frequency (class 1 = 67%; 37% in diploid cells in which *AXL1* is not expressed) (Table 1). This evidence strongly suggests that *AXL1* is a key morphological determinant for the yeast budding pattern. However, the total promoter activity of *LEU2* on high-copy vector in *a/a* cells was about 10 times higher than that of *AXL1* on low-copy vector in *a* cells (luciferase assay was used as a reporter gene; data not shown). Thus, this result may be due to overexpression rather than only ectopic expression.

The predicted amino-acid sequence of *AXL1* contains regions showing extensive similarity to domains of human and *Droso-*

TABLE 1 Quantitative analysis of bud-site selection by wild-type strains, mutant strains defective in *AXL1*, *BUD4* and *BUD5* genes, and wild-type diploid strains containing the *AXL1*-expressing plasmid

Strains	Relevant genotype	Class		
		1	2	3
				
DBY747-SB92DL	<i>MATa</i>	93	1	6
YAF208	<i>MATa/a</i>	35	7	58
CPY20	<i>MATa</i>	32	8	60
YAF203	<i>MATa</i>	35	5	60
YAF204	<i>MATa</i>	7	69	24
YAF201	<i>MATa</i>	8	70	22
YAF209	<i>MATa</i>	34	7	59
DBY746-SB92DL	<i>MATa</i>	95	1	4
YAF210	<i>MATa</i>	34	4	62
YAF208 [YEU3]	<i>MATa/a</i>	37	3	60
YAF208 [YEU- <i>AXL1</i> Lp]	<i>MATa/a</i>	67	1	32

Disruption of the *ACE2* gene^{10,11} causes a defect in cell separation. Although *ace2* strains with axial budding produce hemispherical and lustrous colonies with smooth circular outlines, *ace2* mutants with bipolar budding produce rugged and lusterless colonies with notched outlines. An *axl1-1* mutant (CPY20) with bipolar budding was isolated from an *ace2* strain. CPY20 contained a mutation (not *sir* mutation¹²), *axl1-1*, so named because the wild-type allele is necessary for the axial-budding pattern. Each cell with a single bud and a single bud scar was assigned to one of three classes¹³. Cells of three classes are schematically shown. The open and hatched ovals indicate mother cells and buds, respectively; bud scars are depicted as small rings. The caps covering an area around each end of mother cells are shaded. Cells of class 1 would have a single bud and a single scar both in one cap at one end. Cells of class 2 would have one bud or scar in the cap at one end, and the other scar or bud not in the caps. Cells of class 3 would have one bud or scar in the cap at one end, and the other scar or bud in the cap at the other. Class 1 is characteristic of the axial pattern (also found in bipolar pattern); class 2 is characteristic of a random pattern; class 3 is characteristic of a bipolar pattern. At least 300 cells were scored for each strain. Numbers indicate the percentage of cells in each class. Strains used in this analysis were: DBY747-SB92DL, *ace2::LEU2* in DBY747; DBY746-SB92DL, *ace2::LEU2* in DBY746; YAF208, a cross between DBY747-SB92DL and DBY746-SB92DL; CPY20, *axl1-1* in DBY747-SB92DL; YAF203, *axl1-Δ1::URA3* in DBY747-SB92DL; YAF204, *bud5::HIS3* in DBY747-SB92DL; YAF201, *axl1-Δ1::URA3 bud5::HIS3* in DBY747-SB92DL; YAF209, *MATa bud4* in DBY747-SB92DL and YAF210, *axl1-Δ1::URA3* in DBY746-SB92DL. Square brackets denote the containing plasmid: YEU3 vector (pUC13+*URA3*+2 μm ori DNA); YEU-*AXL1*Lp which has a fusion of the *LEU2* promoter and *AXL1* ORF in YEU3.

phila insulin-degrading enzymes (IDEs)^{3,4} which may play a role in the cellular processing of insulin, and *E. coli ptr* gene product (Ptr)⁵ that could hydrolyse the B-chain of insulin as well as human IDE^{8,9} (Fig. 3c). The close similarity of four proteins among distantly related species such as *E. coli*, *S. cerevisiae*, *Drosophila* and human, suggests a common ancestor. It is thus possible that Axl1 functions as a protease, in a manner analogous to IDE. The identity values (%) for each pair-wise alignment are given in Fig. 3d. The sequences of the human IDE and *E. coli* Ptr can be aligned, except for one large gap in their amino-terminal regions (Fig. 3e). In contrast, the nucleotide sequence of *AXL1* predicts many additional amino-acid residues that cannot be aligned (Fig. 3e). The rather low similarity and the many insertions of the additional amino-acid residues suggest that the Axl1 may function as a protease, but that it might have diverged

from others. Axl1 does not include a signal peptide, as found in *E. coli* Ptr.

This study shows that *AXL1* is a haploid-specific gene and that ectopic expression of *AXL1* allowed *a/a* cells to exhibit an axial budding pattern. Thus, Axl1 is likely to be a key morphological determinant that converts the bipolar budding to axial. Although the structures and roles of the Bud3 and Bud4 are unknown, they may contribute to the establishment of axial

FIG. 2 Expression of *AXL1*. a–f, Phenotypes of strains defective for *AXL1* (a, b) and epistasis relationship of *bud5* and *axl1* null mutations (c–f). Cells were treated with Calcofluor¹⁴ to stain bud scars. Fluorescence micrographs of strain DBY747-SB92DL (*ace2*) (a); CPY20 (*ace2 axl1-1*) (b); DBY747 (wild-type) (c); YAF203 (*axl1-Δ1::URA3*) (d); YAF204 (*bud5::HIS3*) (e); and YAF201 (*axl1-Δ1::URA3 bud5::HIS3*) (f). These strains are isogenic except at the *ACE2*, *AXL1* and *BUD5* loci. g, Northern analysis of the *AXL1* gene. RNAs from the following strains were electrophoresed, blotted and probed with *AXL1* DNA (*MluI*–*EcoRV* fragment) and *LYS2* DNA (*XhoI*–*EcoRV* fragment) as a control for cell-type-nonspecific gene: lane 1, DBY747 (*MATa*); lane 2, DBY746 (*MATa*); and lane 3, YAF106 (*MATa/a*). DBY746 (*MATa his3Δ ura3-52 leu2-3, 112 trp1-289*) and DBY747 (*MATa his3Δ ura3-52 leu2-3, 112 trp1-289*) were wild-type strains obtained from the Yeast Genetic Stock Center, California. YAF106 is the result of a cross between DBY746 and DBY747. Total RNA was isolated from wild-type strains. Poly(A)⁺RNA (8 μg) of each cell type was resolved by electrophoresis on a 1.2% agarose gel. Hybridization was as described elsewhere¹⁵. Genotypes of other strains used for this study are shown in Table 1 legend.

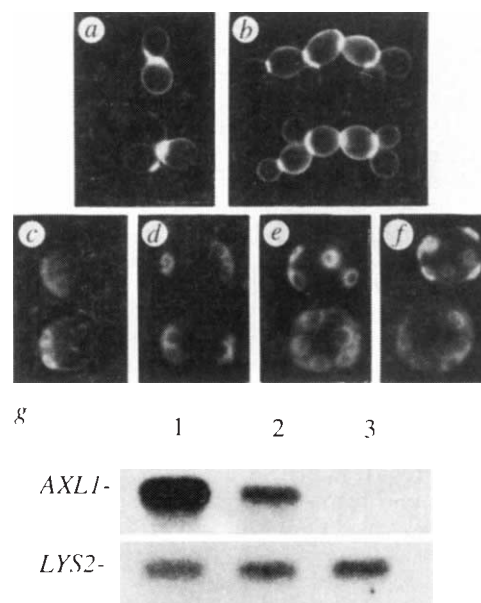
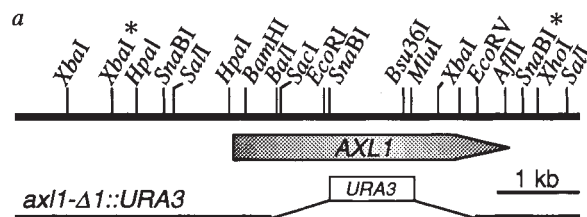


FIG. 3 Restriction map, nucleotide and predicted amino-acid sequences of *AXL1*; similarity of *Axl1* with human and *Drosophila* IDEs and *E. coli* Ptr; pair-wise sequence comparison and schematic representation of these proteins. a, Restriction map of *AXL1*. The nucleotide sequence from the *XbaI* to the *SnaBI* sites marked by asterisks was determined (DDBJ accession number D17787). The position of ORF and structure of the fragment used to disrupt *AXL1* are noted. b, Nucleotide sequence of 5' upstream region of *AXL1* with the N-terminal amino-acid sequence of *Axl1*. Possible $\alpha 1$ - $\alpha 2$ repression sites (5'-ATGTTNNNNNTACATCA-3')¹⁶⁻¹⁸ and two *HpaI* sites are underlined. c, Alignment of the amino-acid sequences of human and *Drosophila* IDEs^{3,4}, yeast *Axl1* and *E. coli* Ptr⁵. White and shaded letters indicate identical and conserved residues, respectively. Dashes indicate gaps to produce optimal alignment. d, Pairwise sequence comparison. Alignments and calculations were done using the FASTA and ODEN programs¹⁹. e, Schematic representation of *E. coli* Ptr, human IDE and yeast *Axl1*. Regions that have strong similarities (shaded boxes) and no similarity (black boxes) are noted.

METHODS. To clone the *AXL1* gene, the *axl1-1* strain was transformed using a yeast genomic library¹⁵. A plasmid (pSH1CU) that complements the *axl1* defect was isolated. A 1.4 kb *EcoRI*-*XbaI* fragment of the insert was subcloned into integrative vector Ylp5²⁰ that contains a *URA3* marker. This construct was used to direct integration by homologous recombination into CPY20 (*axl1-1*). One of the transformants (*axl1*⁻ *URA*⁺) was crossed with a wild-type strain defective in *URA3*. In all 21 tetrads, the *URA3* marker segregated with the bipolar pattern, confirming genetic linkage between the mutant locus and the cloned DNA. For gene disruption, the *EcoRV*-*BamI* fragment was replaced with the *URA3* marker. This was used to replace the wild-type *AXL1*²¹ gene. Disruption was confirmed by Southern analysis.



b

TCTAGAAGGATGACTAATCTATTGTTTCCATTGCTAGCATGGTAAATTAATACATT 56
 TTTGAAATTTGCTGCTAGGGTACAATACCATGCAATGTTTATAATATAAATCAAAATGACA 116
 GAGATTCCCTAAAAGCTAAAACCAATCCATTGATAATATAAGTAGGTTGCTCAATTCAC 176
 CGCAGATTCAATGTAAGTATTGATCTCCATAAATGTTAACTTCAGGTTGATATTCTGTTA 236

HpaI

CAAAAATCTTAGTTTTCCTTACTATCTGAACGGAATGCTCAACATTTTCTCTATT 296
 CTACAAATGTACAAATGTCACGTAACAGTTTGTGATTTTACAGTGGAACATTTT 356
 ACTTGTGTGGCAATAGTACATGCGCAGATAATCAAAAACCATTTTCCGACATTATTTCT 416
 GACAATTTGCTTGAATTTGCTGAACACTTTTCGAGGTACATTCTGATAAAGAGTGCATTAT 476
 TAGTTATTTAGAGTATATTAAGACATCTTCTCGAAGATATATAAATCGTAAGGAAACA 536
 AAAATTTCCGGGATAACAAAAAGTTTCTTCTTTTACGTATTAAATGGTCTATTTCATCGA 596
 TCCATTTCCTATCAATCTTATATATATCAACTTTTGCAATTTCTGCTTCCACCAAG 656
 TCGACGGCTGTTTCTCATCGTTTATGTAATATCAATACCGCATGTATGACATTTATATCAGT 716
 GGCAATGAGCGCTAGTCCATGAATAGGAGTTTAACTCCAACATACGGAACATTCGCATTA 776
 TCGCACACGTAAGACTCGATATATTAAGGAAGGAATTTCCGAGTTGGATTAAATAAGGAA 836
 AAATAAAATATAGTGTGGAAATATAGTTGCTTCAGAATAATATCAATATAATATAA 896
 CGTATATAGTTAGTATACCGGTATATTCGAGTACGCATTAATTCAGCAGTATGACTTTC 956
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 GCGGAGTTACTTGTATCGCGTATTTTACATGCTTTTTCCTGCAAGAAAAATGAAAC 1436
 ACTTATTACACGATTTTCAGGATAGTTTACGCTGGTGGTATGCAAGACATTTTAAACCC 1496

HpaI

TTTGTTTATCTCTTTTGTGCTTGTATTATATAATAATGTGCTGGCGTTAAAAATA 1556

GCAACTGAATAAGTTTCTTACTGATGCTCTGAGAGTAACATAATATYGAAGTCTCG 1616
 M S L R E V T N Y E V S 12
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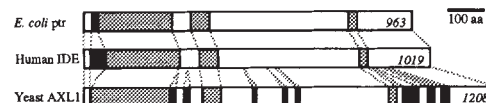
c

Human IDE	M-(59aa)-	-----	103
<i>Drosophila</i> IDE	M-(73aa)-	-----	117
Yeast <i>AXL1</i>	M-(19aa)-	-----	63
<i>E. coli</i> ptr	M-(39aa)-	-----	83
104	-----	-----	156
118	-----	-----	170
64	-----	-----	123
84	-----	-----	136
157	-----	-----	216
171	-----	-----	230
124	-----	-----	183
137	-----	-----	196
217	-----	-----	276
231	-----	-----	290
184	-----	-----	243
197	-----	-----	253
277	-----	-----	377
291	-----	-----	391
244	-----	-----	390
254	-----	-----	355
378	-----	-----	1019
392	-----	-----	1031
391	-----	-----	1208
356	-----	-----	962

d

	<i>Drosophila</i> IDE	<i>E. coli</i> ptr	Yeast <i>AXL1</i>	(%)
Human IDE	45	26	19	
<i>Drosophila</i> IDE		26	19	
<i>E. coli</i> ptr			21	

e



budding with Ax11. We have recently identified a mutation (*rax1*) that can convert the budding pattern of *ax11* null strain from bipolar to axial (unpublished data). The putative amino-acid sequence of Rax1 contained several conserved residues of B-chains of the insulin-related hormone superfamily (A.F. *et al.*, unpublished data). Characterization of this gene may provide new insight into the control of bud-site selection. □

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Interleukin-2-mediated elimination of the p27^{Kip1} cyclin-dependent kinase inhibitor prevented by rapamycin

Jamison Nourse*, Eduardo Firpo†, W. Michael Flanagan‡§, Steve Coats†, Kornelia Polyak||, Mong-Hong Lee||, Joan Massague||, Gerald R. Crabtree*‡ & James M. Roberts†

* Program in Cancer Biology and † Howard Hughes Medical Institute, Stanford University Medical School, Stanford, California 94305, USA
‡ Department of Basic Sciences, Fred Hutchinson Cancer Center, 1124 Columbia Street, Seattle, Washington 98104, USA
|| Cell Biology and Genetics Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA

THE cyclin-dependent kinase (Cdk) enzymes, when associated with the G1 cyclins D and E, are rate-limiting for entry into the S phase of the cell cycle^{1,2}. During T-cell mitogenesis, antigen-receptor signalling promotes synthesis of cyclin E and its catalytic partner, Cdk2, and interleukin-2 (IL-2) signalling activates cyclin E/Cdk2 complexes³. Rapamycin is a potent immunosuppressant which specifically inhibits G1-to-S-phase progression, leading to cell-cycle arrest in yeast and mammals^{4–7}. Here we report that IL-2 allows Cdk activation by causing the elimination of the Cdk inhibitor protein p27^{Kip1}, and that this is prevented by rapamycin. By contrast, the Cdk inhibitor p21 is induced by IL-2 and this induction is blocked by rapamycin. Our results show that p27^{Kip1} governs

Cdk activity during the transition from quiescence to S phase in T lymphocytes and that p21 function may be restricted to cycling cells.

Rapamycin prevents the activation of the late-G1 cyclin E/Cdk2 kinase in the T-cell clones D10.G4.1 (Fig. 1a) and CTLL-2 (ref. 8) and primary T lymphocytes (data not shown). The inactivity of cyclin E/Cdk2 in rapamycin-treated cells is not due to an inhibition of the expression of cyclin E or Cdk2 (Fig. 1b), to an inability of cyclin E to associate with Cdk2 (Fig. 1c), or to an inhibition of Cdk-activating kinase (CAK) activity (Fig. 1d).

We find that extracts from both IL-2-starved and IL-2/rapamycin-treated cells contain an inhibitory activity for cyclin E/Cdk2 kinase whereas IL-2-induced controls do not (Fig. 2a, b). Examination of synchronized IL-2-induced and IL-2/rapamycin-treated cells suggest that an inhibitory activity is present for up to 3 h after IL-2 induction and is gradually removed after longer exposure to IL-2 in the absence of rapamycin (Fig. 2c). This inhibitory activity could be titrated out by the addition of supraphysiological amounts of exogenous cyclin E and cyclin E/Cdk2 but not Cdk2 alone (Fig. 2d).

A heat-stable inhibitory activity is directly associated with cyclin E/Cdk2 complexes (Fig. 2e). Surprisingly, the inhibitory activity is also detected in cyclin E/Cdk2 complexes isolated from IL-2-induced cells. Accurate analyses designed to measure the amount of inhibitor present in cyclin E/Cdk2 complexes under these various conditions show that its levels are lower in IL-2-induced cells than in arrested cells (Figs 3c, 4).

Mammalian Cdk activities are inhibited *in vitro* by the associated proteins p16, p21 and p27^{Kip1} (refs 9–15). A heat-stable doublet corresponding to a relative molecular mass of 27,000 is found associated with cyclin E immunoprecipitates of D10 cell extracts, which, like p27^{Kip1}, binds preferentially to recombinant cyclin E/Cdk2 rather than to cyclin E or Cdk2 alone (Fig. 3a). This doublet comigrates with the doublet in p27^{Kip1} immunoprecipitates (Fig. 3a). Proteolytic mapping of these 27K bands establishes that they are identical to p27^{Kip1} (Fig. 3b). Immuno-

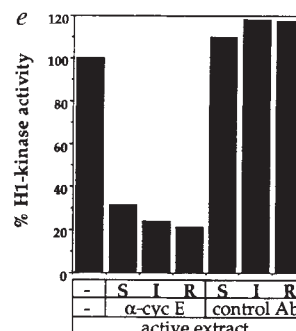
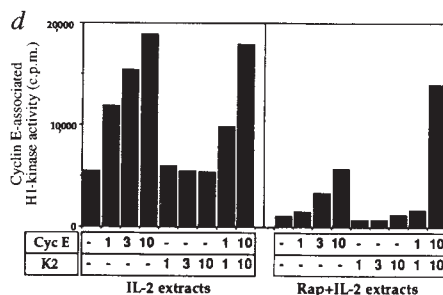
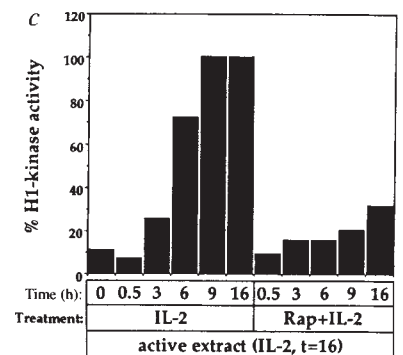
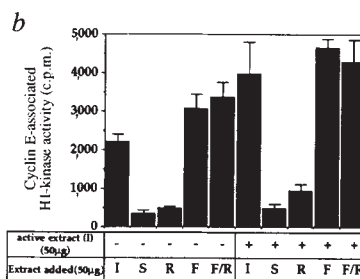
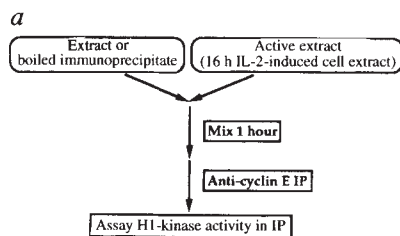
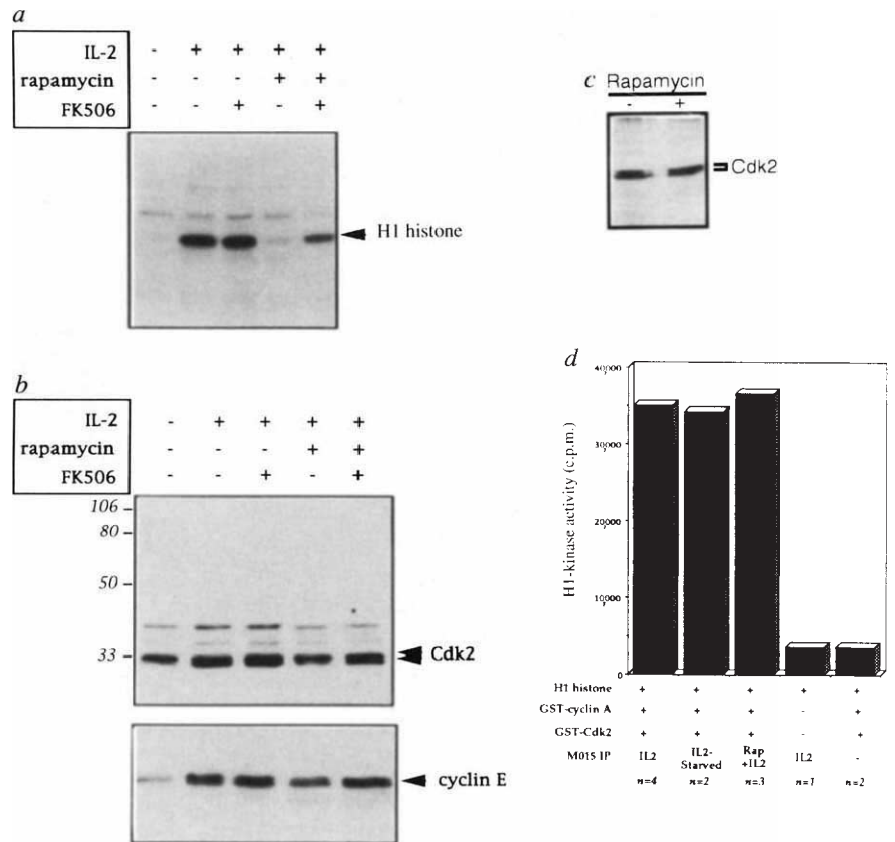
FIG. 2 Rapamycin-treated cells contain a titratable and heat-stable inhibitor which is directly associated with cyclin E/Cdk2 complexes. a, Experimental design used in b, c and e. Inhibitory activity is measured in extracts or immunoprecipitates by mixing with active extracts and subsequently measuring cycling E-associated H1 kinase activity. b, Kinase activity in active extracts after mixing with extracts from IL-2-starved cells (S), IL-2-induced (I), IL-2/rapamycin-treated (R), IL-2/FK506-treated (F) or IL-2/rapamycin/FK506-treated (F/R). The experiment was performed in triplicate. c, Kinase activity in active extracts after incubation with an equal amount of extracts of cells collected at various times (in hours) after treatment. d, Cyclin E-associated H1-kinase activity in extracts after incubation with cyclin E and/or Cdk2-HA Sf9 lysates added in multiples (1, 3 or 10 times) of the physiological levels of cyclin E and Cdk2. Data are representative of two independent experiments. e, Boiled cyclin E or control immunoprecipitates from the indicated cell extracts are assayed as for a. This experiment demonstrates that a Cdk inhibitor is present in cyclin E immune complexes from both growing and arrested cells. This protocol is not designed to quantify the amount of inhibitor present in each condition, because of difficulties in reconstituting the physiological ratio of cyclin E to inhibitor after immunoprecipitation from cell extracts.

METHODS. D10 cell extracts were mixed for 1 h at 37 °C, then cyclin E was immunoprecipitated and assayed for H1-kinase activity as described previously³. ³²P incorporation into H1 histone was quantified using AMBIS Colorprobe software (version 2.12). Sf9 lysates of cyclin E and Cdk2-HA and D10 extracts prepared in NP-40 buffer was described previously³. Physiological concentrations of cyclin E and Cdk2 were determined by comparative western blotting. Washed cyclin E or control immunoprecipitates were resuspended in 50 µl N-P40 buffer containing 1 mg ml⁻¹ aprotinin and heated at 97–100 °C for 3–5 min. After adding 50 µl 2 mg ml⁻¹ BSA in NP-40 buffer, the Sepharose and coagulated proteins were removed by centrifugation and the soluble fraction assayed.

§ Present address: Gilead Sciences, 346 Lakeside, Foster City, California 94404, USA

FIG. 1 Rapamycin inhibits cyclin E/Cdk2 kinase activity without significantly inhibiting cyclin E or Cdk2 expression, cyclin E/Cdk2 association or CAK activity. *a*, Cyclin E-associated H1-kinase activity in IL-2-starved or IL-2-stimulated D10 cells in the absence or presence of the drugs rapamycin and FK506. *b*, Immunoblot analysis of whole D10 cell extracts with anti-Cdk2 (upper panel) or anti-cyclin E antibodies (lower panel). *c*, Anti-Cdk2 immunoblots of α -cyclin E immunoprecipitates from peripheral blood T-lymphocyte extracts. *d*, *In vitro* assay of CAK activity from extracts of IL-2-starved and IL-2-stimulated (IL2) D10 cells in the presence of rapamycin (Rap + IL2).

METHODS. D10 cells were synchronized by washing the cells of IL-2-containing medium and subsequently culturing in IL-2-deficient medium for 20–22 h. Cells were collected (IL-2-starved) or were stimulated with recombinant IL-2 (DNAX) in the presence or absence of either 2 ng ml⁻¹ rapamycin or 2 ng ml⁻¹ FK506 or 2 ng ml⁻¹ rapamycin and 2,000 ng ml⁻¹ FK506 and collected 16 h after stimulation. Rapamycin's inhibition of proliferation is mediated by the intracellular binding protein, FKBP. As FK506 also binds FKBP with similar affinity, it reverses the effects of rapamycin by competitive binding when FK506 is present at 1,000-fold molar excess^{22,23}. Immunoprecipitation, kinase assay and immunoblotting were performed as described³. Peripheral blood T lymphocytes were purified and stimulated as described³. CAK activity was immunoprecipitated from extracts with the anti-M015 antibody¹⁷. Washed immunoprecipitates were mixed with 1 μ g of both recombinant GST-cyclin A and GST-Cdk2 fusion proteins in 30 μ M ATP and 15 mM magnesium acetate in 7.5 μ l. After 30 min at room temperature, 2.5 μ l H1 histone mix containing [γ -³²P] ATP was added and incubated as described¹⁷.



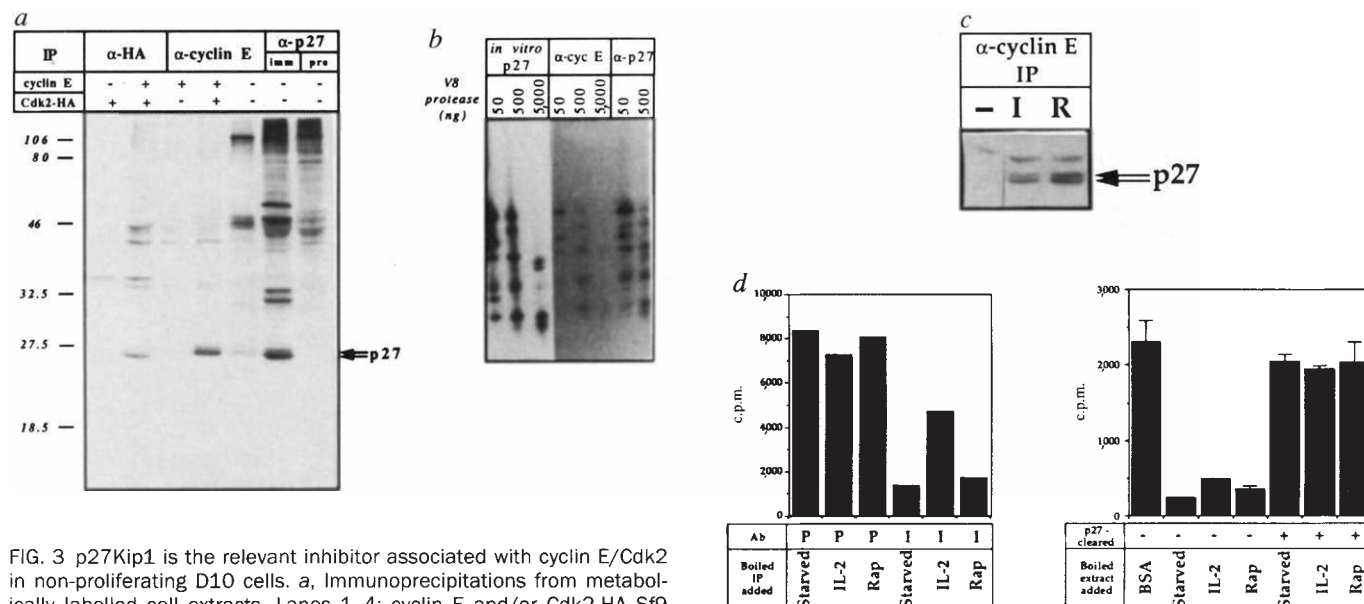


FIG. 3 p27Kip1 is the relevant inhibitor associated with cyclin E/Cdk2 in non-proliferating D10 cells. **a**, Immunoprecipitations from metabolically labelled cell extracts. Lanes 1–4: cyclin E and/or Cdk2-HA Sf9 lysates were added to the uncoagulated fraction of boiled extracts and immunoprecipitated with 12CA5 (anti-HA) or anti-cyclin E antibodies. Lanes 5–7: immunoprecipitations from native extracts with anti-cyclin E, anti-p27Kip1 or preimmune sera. **b**, V8 protease mapping of the p27 bands isolated from cyclin E or p27Kip1 immunoprecipitates and the *in vitro* translated p27Kip1 protein were performed as described²⁴. **c**, Anti-p27Kip1 immunoblot of cyclin E immunoprecipitation from the indicated cell extracts. The control immunoprecipitate labelled as a dash was performed without cell lysate, but with primary (anti-cyclin E) antibody in the immunoprecipitation. **d**, p27Kip1 antibodies remove the inhibitor from rapamycin-treated cell extracts. Boiled preimmune (P) or anti-p27Kip1 (I) immunoprecipitates from cell extracts (left panel) and boiled extracts that had been precleared of p27Kip1 with anti-p27Kip1 or preimmune sera (right panel) were assayed for inhibitory activity as described for Fig. 2a; the experiment was done in triplicate. Anti-p27

antibodies raised against bacterially expressed C-terminal domain of p27 that is divergent from p21 produced the same results. **METHODS.** Rabbit anti-p27Kip1 antibodies were generated against purified His-tagged, full-length mouse p27Kip1 protein¹⁴. These antibodies are specific for p27Kip1 as p21 is not detected in immunoblots or in p27Kip1 immunoprecipitates of metabolically labelled cells extracts (data not shown). Cells cultured in the absence of IL-2 for 20 h were metabolically labelled for the last 15 h with ³⁵S-promix (Amersham). p27Kip1 was removed from 100 µg extract with affinity-purified anti-p27Kip1 or preimmune serum, and the resulting extract was boiled to remove any remaining cyclin E and to liberate p27Kip1. In a separate experiment (scaled up twofold), immunoprecipitates were assayed as described for Fig. 2d.

blotting cyclin E immunoprecipitates reveals that, by comparison with rapamycin-arrested cells, p27^{Kip1} declines by about 50% in cyclin E complexes from IL-2-induced cells (Fig. 3c); when peripheral blood T-lymphocyte extracts are analysed, p27^{Kip1} is only associated with cyclin E from rapamycin-treated and IL2-starved cells (data not shown).

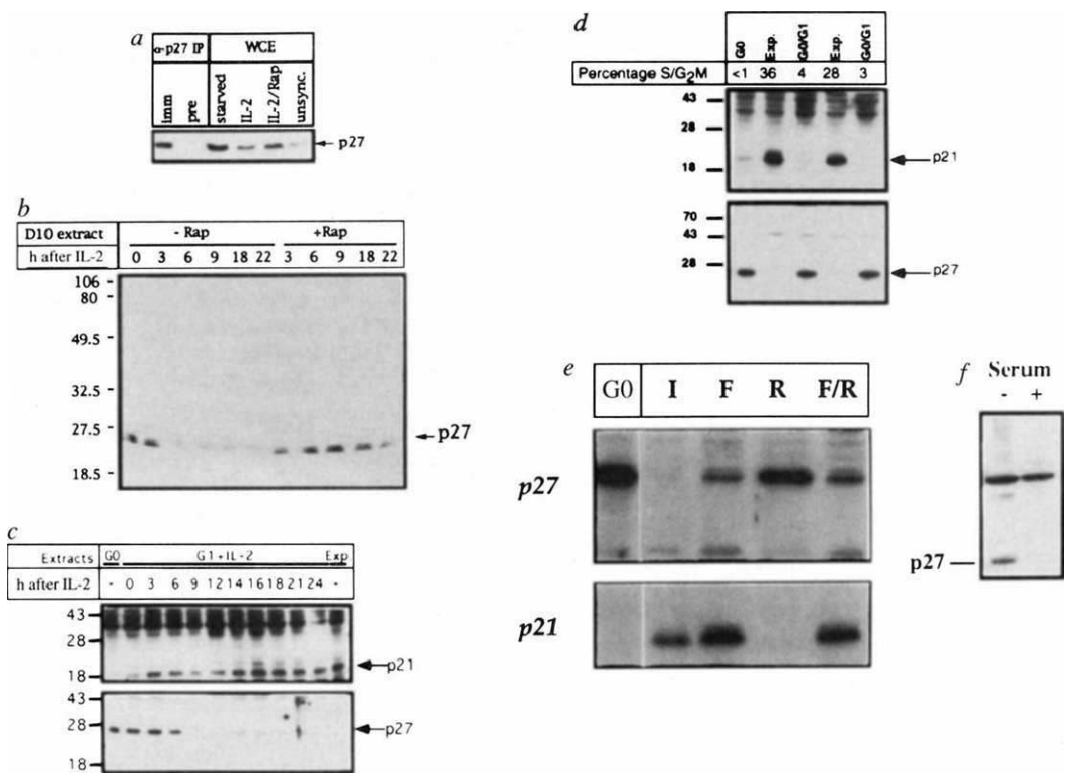
Antibodies specific for p27^{Kip1} clear extracts of all the inhibitory activity in IL2-starved and rapamycin-treated D10 cells (Fig. 3d), suggesting that raised levels of p27 account for essentially all of the inhibitory activity detected in arrested cells. Likewise, RNase protection assays demonstrate that IL-2-starved or rapamycin-treated D10 cells do not have increased levels of p21 messenger RNA compared with IL-2-induced cells (data not shown).

Immunoblotting whole-cell extracts shows that IL-2 induction gradually reduces the amount of p27^{Kip1} and, significantly, that rapamycin prevents removal of p27^{Kip1} (Fig. 4a, b). Some p27^{Kip1} remains in the IL-2-stimulated population, which could be due to contaminating unstimulated cells because 18% of synchronized IL-2-treated cells do not traverse S phase (versus 5% of unsynchronized cells) after 24 h, as monitored by BrdU incorporation (data not shown). Alternatively, IL-2 stimulation may not completely remove p27^{Kip1} in D10 cells. Indeed, p27^{Kip1} is present at low levels in unsynchronized cycling cells (Fig. 4a). The observations that IL-2-induced cells enter S phase and have significant cyclin E/Cdk2 kinase activity, despite the presence of some inhibitor, suggests that p27^{Kip1} levels function as a threshold and that this threshold has been sufficiently lowered in D10 cells to allow cyclin E/Cdk2 function.

Three experiments show that the mechanism of Cdk2 inhibition by p27^{Kip1} is not the inactivation of CAK, which activates Cdk2 by phosphorylation on Thr 160 (refs 16–18). First, the faster-migrating Thr-160-phosphorylated isoform of Cdk2 (ref. 19), is associated with cyclin E in relatively the same abundance in rapamycin-blocked and proliferating cells (Fig. 1c). Second, immunoprecipitation with antibodies specific for the catalytic subunit of CAK (anti-M015)¹⁷ show that CAK activity is equally abundant in extracts of both untreated and rapamycin-treated IL-2-stimulated cells (Fig. 1d). Third, purified p27^{Kip1} does not inhibit purified CAK *in vitro* (R. Sheaff, R. Fisher, D. Morgan and J.M.R., unpublished result). Thus, p27^{Kip1} directly blocks the activity of the Thr-160-phosphorylated cyclin E/Cdk2 complex, as found previously *in vitro*^{13,14}.

The results obtained in D10 cells were extended and confirmed in peripheral blood T lymphocytes. Immunoblot analysis of peripheral blood T lymphocytes at various times after IL-2 stimulation reveals that p21 increases as p27^{Kip1} decreases (Fig. 4c), indicating that the previously described p27 inhibitor in T lymphocytes is indeed p27^{Kip1} (ref. 3). The inverse relationship between p27^{Kip1} and p21 is confirmed by analysis of peripheral blood T lymphocytes undergoing reiterated cycles of proliferation and quiescence. In each cycle proliferation is induced by anti-CD3 antibodies and IL-2, whereas cells are arrested by withdrawal of IL-2 (Fig. 4d). IL-2-mediated removal of p27^{Kip1} is blocked significantly by rapamycin but not by FK506, indicating that it is a TOR/FRAP/RAFT-dependent event^{5,7} and not related to the calcineurin pathway activated by antigen-receptor signalling²⁰ (Fig. 4e). The repression of p27^{Kip1} expression by

FIG. 4 Rapamycin inhibits IL-2-mediated removal of p27^{Kip1} and induction of p21. **a**, p27^{Kip1} immunoblot analysis of preimmune and anti-p27^{Kip1} immunoprecipitates and of whole-cell extracts (WCE). **b**, p27^{Kip1} immunoblot of extracts from synchronized D10 cells stimulated in the absence or presence of rapamycin and collected various times (in hours) after IL-2-induction. **c**, p27^{Kip1} and p21 immunoblots of freshly isolated (G0) and IL-2-induced G1 phase peripheral blood T lymphocytes collected at various times (in hours) (G1+IL-2) after IL-2 treatment and exponentially proliferating (Exp). **d**, p27^{Kip1} and p21 immunoblots of primary T lymphocytes (G0) or long-term cultures of peripheral blood T cells that are resynchronized by IL-2 deprivation (G0/G1) and subsequently restimulated with IL-2 (Exp.). **e**, p27^{Kip1} and p21 immunoblots of G0, IL-2 stimulated (I) or IL-2 stimulated with 4 ng ml⁻¹ FK506 (F), rapamycin (R) or 4 ng ml⁻¹ rapamycin and 4 ng ml⁻¹ FK506 (F/R). **f**, p27^{Kip1} immunoblot of whole-cell extracts of serum-starved (-) or serum-stimulated (+) primary human fibroblasts. METHODS. 10⁸ human primary T lymphocytes were isolated and purified and stimulated as described³. For **d**, stimulation was achieved by plating cells on immobilized anti-CD3 antibodies in complete RPMI medium³ containing 1 µg ml⁻¹ anti-CD28 antibodies. After 2 days at 37 °C, cells were transferred to a new flask with fresh complete RPMI containing



1 µg ml⁻¹ anti-CD28 and 100 U ml⁻¹ recombinant human IL-2. After 3 more days in culture, an aliquot was taken (Exp) and the rest were washed and incubated for 24 h in complete RPMI. This washing process was repeated for a total of 3 days and an aliquot was taken (G0/G1) and the rest restimulated with immobilized anti-CD3 antibodies as before. Confluent T-6 fibroblasts (gift from T. Norwood) were serum-starved for 48 h and restimulated with 10% fetal bovine serum for 24 h.

extracellular mitogens is not restricted to lymphocytes: serum growth factors also repress p27^{Kip1} expression in primary human diploid fibroblasts (Fig. 4f).

The inhibition of cyclin E/Cdk2 by p27 is likely to reflect a more general inhibition of G1 cyclin-Cdk complexes in rapamycin-treated cells because (1) rapamycin does not inhibit expression of Cdk4 or Cdk6, or of their partners, cyclins D2 or D3; and (2) p27^{Kip1} associates with Cdk6 as T cells depart from quiescence, and this association persists in rapamycin-treated cells (data not shown).

We have demonstrated that in T cells leaving a quiescent state, IL-2 causes a decrease in p27^{Kip1}, allowing Cdk2 activation and entry into S phase. In contrast, we find that IL-2 induces p21,

and that p21 expression persists in cycling cells. But cycling D10 cells continue to express some p27^{Kip1}, albeit at substantially lower levels than quiescent cells (Fig. 4a). Therefore in normally cycling cells, both p27^{Kip1} and p21 may together establish a threshold and that must be overcome for Cdk activation to occur in G1. Increasing the levels of p21 by intracellular 'checkpoint' signals, such as those resulting from DNA damage, or regulating p27^{Kip1} by extracellular or intracellular signals such as cyclic AMP (ref. 21), can arrest the cell cycle by increasing a threshold that cannot be overcome by physiological concentrations of cyclin-Cdk. Our data suggest that p27^{Kip1} may be the primary regulator of Cdk activity as cells enter and leave the quiescent state, whereas p21 may primarily regulate Cdk activity in cycling cells. □

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Damage report for *BRCA1*

Three groups have together uncovered more than 20 distinct mutations in families with hereditary breast and ovarian cancer, offering important clues about the function of *BRCA1*.

Two months ago, on 7 October, a beautiful article by Mark Skolnick and 44 colleagues describing the long awaited discovery of the gene for hereditary breast and ovarian cancer, *BRCA1*, was published in *Science*¹. That same day the complete nucleotide sequence of *BRCA1* was unveiled in the public databases. Instantly, several groups began designing their own oligonucleotide primers to analyse *BRCA1* in high-risk families using the polymerase chain reaction. The results of some of this frenetic activity can be found in three papers²⁻⁴ in this month's issue of *Nature Genetics*. Together, they provide a considerable amount of new information about this infamous gene.

The initial evidence for *BRCA1* having been cloned hinged on the discovery of five inherited mutations in high-risk families¹, coupled with the presence of four germline mutations in a series of other cancer patients who were not known to have an extensive family history of the illness⁵. Just as for cystic fibrosis five years ago, the main priority for breast-cancer researchers has been to compile a comprehensive list of mutations, with two main goals in mind: to shed light on the function of *BRCA1* by discerning a pattern in the genetic lesions in different families, and to estimate how many different aberrations in *BRCA1* can cause cancer, which has obvious implications for screening other high-risk families.

In the new findings, three groups, led by Barbara Weber (University of Pennsylvania), Steven Narod (McGill University) and Mary-Claire King (Berkeley) screened a total of 100 families with multiple cases of breast (and often ovarian) cancer for signs of mutations in *BRCA1*, and were successful in 31 cases. This rate may seem low until one recalls that the recently mapped *BRCA2* locus also gives rise to a considerable number of hereditary cancers, and current methods of gene screening are not 100 per cent accurate, especially for large multi-exon genes such as *BRCA1*.

Of these 31 mutations, 22 are distinct, constituting a complex array of frameshift and missense mutations scattered along the length of the gene²⁻⁴. Most result in the premature truncation of the gene product, although whether these act in a recessive or a dominant-negative manner is not yet known. Narod's group found two mutations in patients from four separate Canadian breast-cancer families. These families are not thought to be related, but they do share common haplotypes around *BRCA1*, suggesting a possible founder effect in some cases³. Efforts to seek a correlation between genotype and phenotype are probably premature, but already there are some curious offerings. In one of King's families, for example, a nonsense mutation⁴ crops the last 11 amino acids from the C terminus of the *BRCA1* gene product out of a total of 1,863. On the face of it, this might be considered to be a relatively mild change, and yet women in this family have developed breast cancer at a very early age, in their 20s and 30s.

A handful of *BRCA1* mutations switch one amino acid for another, but although these tend not to reveal much about the function of the protein, there seem to be two prominent exceptions^{2,4}. The only recognizable domain in the *BRCA1* protein is a C3HC4 zinc-finger domain close to the N terminus. Two substitutions (one of which occurs in two unrelated families) replace one of the last two cysteine residues in this motif, indicating that this is a functionally important part of the protein and fuelling speculation that the protein encoded by *BRCA1* acts as a transcription factor.

But the most sobering aspect of these results is the sheer volume of mutations that can destroy *BRCA1*, which will make screening women on a large scale, if and when this becomes appropriate, a technically demanding feat. Recently, several academic and advocacy groups, including the National Breast Cancer Coalition and the American Society for Human Genetics⁶, have gone on record against large-scale *BRCA1* screening, until much more is understood about the biology of *BRCA1* and the consequences of diagnosis and counselling.

Elsewhere in this month's issue, Doug Marchuk and colleagues at Duke University present solid evidence to implicate a transforming growth factor- β (TGF- β) binding protein, endoglin, in a vascular

disorder called hereditary haemorrhagic telangiectasia (HHT)⁷. Patients with HHT are susceptible to frequent bleeding episodes, especially in the nose and gut, but some patients develop pulmonary arteriovenous malformations that can lead to aneurysms and strokes.

Earlier this year, the HHT locus was mapped by two groups to the long arm of chromosome 9 (refs 8, 9). Marchuk's group soon turned its attention to the endoglin gene, and has now described three different deleterious mutations in endoglin in HHT patients. Endoglin is an integral membrane glycoprotein which, in the presence of ligand, associates with the endothelial TGF receptor. Marchuk and colleagues suggest that this signalling process is impaired in HHT patients, leading to abnormal vascular remodelling in response to injury, for example, although the precise cellular mechanism remains to be determined.

Finally, researchers at the Rice Genome Research Program in Tsukuba, Japan, have compiled the most detailed genetic map so far of the 12 chromosomes that make up the 450-million-base-pair rice genome¹⁰. There are many incentives for studying the rice genome, not least of which is the undisputed importance of the plant in forming the staple diet of about half of the world's population. But in addition to developing thorough genetic and expression maps to aid the isolation of genes controlling disease resistance, growth and other agronomically important traits, rice also promises to serve as an extremely useful model organism for other cereals such as maize and wheat¹¹. Of the nearly 1,400 polymorphic DNA markers on the Japanese map, almost 900 are expressed gene fragments, providing an average marker density of one every 300 kilobases. As such, the work serves as an important landmark towards the ultimate goal of determining the complete sequence of the rice genome. **Kevin Davies**

Kevin Davies is Editor of *Nature Genetics*.

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Also in this month's *Nature Genetics*: the gene for X-linked Emery-Dreifuss muscular dystrophy; mutations in a metalloproteinase inhibitor (TIMP3) in Sorsby's fundus dystrophy; assembly of dystrophin-associated proteins in transgenic mice; imprinting of the *mas* oncogene; and 'searching for shared segments' — a linkage disequilibrium approach to gene mapping.

Cell science

On the market in the area of cell biology — a kit for CD8 cell enrichment, an automated cell viability assay system and cultureware that provides anchorage-dependent cells with a surface that supports receptor-mediated cell adhesion.

PIERCE's mouse and human IsoCell **CD8 isolation kits** are affinity chromatography tools for CD8 cell enrichment (*Reader Service No. 100*). The company says that virtually all B lymphocytes and CD4 cells are removed using a negative selection process on affinity columns. And, a large percentage of macrophages and monocytes are also removed because of their nonspecific binding properties. Under optimal conditions, Pierce says that greater than 95 per cent of B and CD4 cells can be removed from lymphocyte preparations.

The BTX ECM 600 **electroporation system** offers two voltage ranges (2.5 kV/500 V) for a variety of applications, an additive capacitor bank, resistance timing and instantaneous monitoring — all within a single unit (*Reader Service No. 101*). With



Electro-cell manipulator — made by BTX.

this model, users can switch between electroporation of prokaryotic and eukaryotic cells at the turn of a knob. The instrument is said to be shock proof, arc proof and short-circuit proof. It is fitted with an internal monitoring system that measures true peak voltage and pulse length. Each ECM 600 system consists of a safety stand, cuvette rack and a package of 50 sterile, disposable cuvettes in a choice of 1, 2 or 4 mm gap sizes. In addition to technical support, BTX provides users with access to its Electronics Genetics Database which contains over 2,500 papers on electroporation and electrofusion data.

■ Systems

The CellStat system, available from CellStat Technologies, is a fully automated, continuous, **noninvasive cell viability assay system** that can work around the clock (*Reader Service No. 102*). It can be used to measure changes in the electrical conductivity of the media created by the presence of a cell culture and its metabolites. These changes are known to have a positive correlation to cell growth: an increase in productivity indicating proliferation. In addition to being

nondestructive and noninvasive, the system requires no reagents for operation and produces no hazardous waste. The test data may be displayed in a number of configurations without interrupting the test, using either the CellPlot application, or for a hard copy report using the Report application in the DOS-based host software.

Cardinal is a new **automatic colony counting system** available from Perceptive Instruments that has been developed for the analysis of bacterial or mammalian cultures across a range of growth media (*Reader Service No. 103*). At the heart of the system is a compact plate viewer with lighting for colonies on transparent and opaque nutrient media. The system is completed by a measurement card inside an industry-standard computer with Cardinal software running under Microsoft Windows. In routine operation, the user enters sample details, after which the plate is analysed with detected colonies highlighted in colour. Problems such as overlapping colonies, irregularly shaped growths, cloudy media and unevenly poured plates are addressed by image processing techniques. Small colonies or debris can be removed from the overall count. Results appear on screen after each measurement and are printed or saved to the computer's hard or floppy disk. Data can be transferred to a spreadsheet program such as Microsoft Excel. Cardinal can also be integrated with bar-code readers and laboratory information management software.

Becton Dickinson Immunocytometry Systems' FACSCount System is a benchtop **clinical system for monitoring patients with HIV infection** (*Reader Service No. 104*). It can be used to obtain absolute counts of CD4, CD8 and CD3 T-lymphocytes. The system is said to require very little specialist knowledge for operation. A three-step method is designed to reduce sample han-



FACSCount System — makes a count of CD4, CD8 and CD3 T-lymphocytes.

dling and greatly reduce the chance of operator error. And, as the system uses whole blood, the need to lyse red cells in blood samples has been eliminated. Once the blood sample is analysed, the system produces a summary of the absolute numbers of CD4, CD8 and CD3 T-lymphocytes, a helper/suppressor ratio and the control results from its built-in printer.

■ Assays

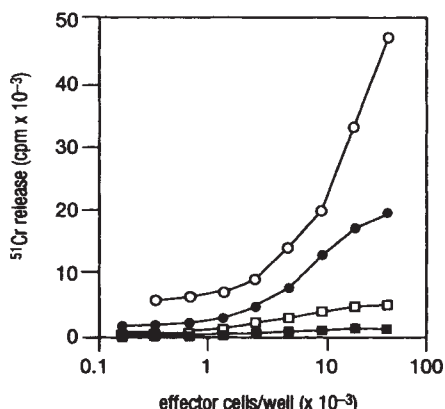
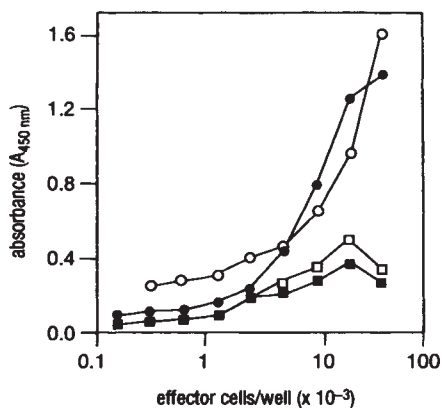
With the new TiterScreen 'sandwich' enzyme immunoassay kits from PerSeptive Diagnostics for the qualitative **determination of multiple human cytokines** in serum, plasma or tissue culture supernatants, results can be determined in less than 3 h



Multiple cytokine EIA screening kit.

(*Reader Service No. 105*). They are as follows: TiterScreen I for the measurement of IL-1 β , IL-6 and TNF α , TiterScreen II for the detection of IL-2, IFN γ and TNF α and TiterScreen III for IL-4, IL-10 and TNF α . Each assay contains one 96 detachable well plate and stable reagents for the qualitative evaluation of three different cytokines using a horseradish-peroxidase enzyme conjugate and TMB substrate for detection at 450 nm. Only 100 μ l of sample is said to be required. The colour-coded wells for each cytokine provide 32 tests per analyte, including standard curve values.

Boehringer Mannheim's new **cellular DNA fragmentation ELISA** can be used by researchers studying two of the most common types of cell death (*Reader Service No. 106*). In apoptosis studies, the ELISA deter-



Cellular DNA fragmentation ELISA (A) in comparison with the ^{51}Cr -release assay (B). 3×10^4 ($\circ$), 1×10^4 ($\bullet$) and 1×10^3 ($\blacksquare$) prelabelled P815 target cells were incubated at different E/T ratios for 4 h. After incubation, 100 μl per well supernatant (=SN) were removed and measured either directly by ELISA (A) or in a gamma-counter (B).

mines the number of BrdU-labelled fragments in the cytoplasm of affected cells. And, in studies into cell-mediated toxicity, it can be used to measure the BrdU-labelled DNA fragments that damaged cells release into the culture supernatant. The assay is

designed to provide a more rapid and non-radioactive alternative to ^{51}Cr -release assays.

Antibodies

Maine Biotechnology Services announces the addition of a number of **sheep polyclonals** to its product line (*Reader Service No. 107*). The new line-up includes ATF-1, cMYC, EGFr, endothelin B-MC2, human GTPase activating protein (GAP), glucose transporter (GLUT), HIV-rev, HIV-TAT, LYN, MAP kinase, RAL-A and SOS1-B6. MBS also has available four new monoclonal antibodies specific for *Mycoplasma* sp., *Mycoplasma pneumoniae* and *Mycoplasma genitalium*. The company, which develops and produces monoclonal antibodies under contract, sells many reagents licensed from universities and national research organizations, as well as those produced by the company.

Biogenesis has introduced a new **anti-body that recognizes sodium glucose transporter 1 (SGLT1)** (*Reader Service No. 108*). This rabbit polyclonal antibody should be of interest to researchers in the field of glucose metabolism. The anti-SGLT1 is said to work at a titre of around 1/500 in western blotting and at greater than 1/30,000 in ELISAs.

Culture systems

ProNectin F Activated Cultureware from Protein Polymer Technologies is designed to provide anchorage-dependent cells with a **culture surface that supports receptor-mediated cell adhesion** and spreading, initiating cell growth (*Reader Service No. 109*). The RGD cell attachment ligand of fibronectin is presented directly on the culture surface in the form of ProNectin F, a genetically engineered cell attachment factor. The product is sold sterile, stable and ready-to-use in dish and multiwell plate formats.

Unipath has introduced a new 2.5-litre **anaerobic jar** for use with the Oxoid atmosphere generation product, AnaeroGen (*Reader Service No. 110*). Called the AnaeroJar, the new jar will hold up to 12



Unipath's new anaerobic jar — with room for 12 Petri dishes.

disposable plastic Petri dishes at a time. The jar has a space-saving design with no external clamps or protruding valves, and an integral, retractable carrying handle. Its lightweight polycarbonate base is secured to the lid by means of four spring-loaded clips. The clips themselves allow self-venting to take place in the unlikely event of a build-up of pressure. A vacuum relief screw permits the inlet of air to the jar, thus overcoming any difficulty in opening the lid which could be caused by the presence of negative pressure. When sealed inside the jar, Oxoid AnaeroGen sachets will produce an atmosphere that contains less than 1 per cent oxygen, supplemented with carbon dioxide. No catalyst or water additions are required.

Two new **human microvascular endothelial cell systems** (providing lung and dermal-derived cells) — EndoPack-MVL and EndoPack-MVd — are available from Clonetics and should be of interest to researchers working on respiratory disease, wound healing, inflammation and neo-vascularization in tumour growth, as well as basic research (*Reader Service No. 111*). EndoPack-MVL provides researchers with one normal human lung microvascular endothelial cell product, proliferating of cryopre-

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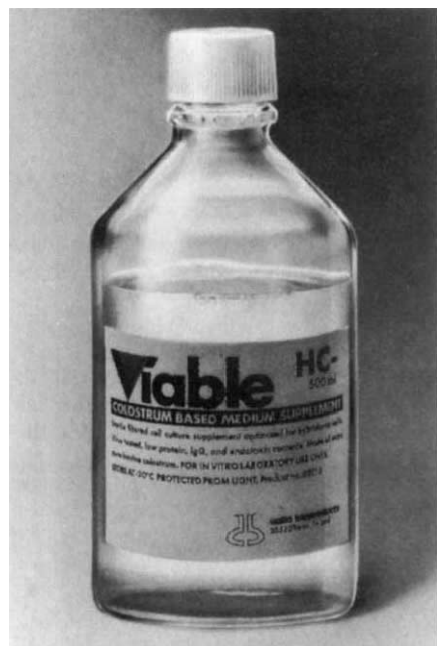
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served (T-25 flask or cryoamp $\geq 500,000$ cells per amp), one 500-ml bottle of EGM-MV, optimized microvascular endothelial cell growth medium and one ReagentPack containing trypsin/EDTA in HBSS, trypsin neutralizing solution and HEPES buffered saline solution. The EndoPack-MVd cell culture system contains the same cell growth medium and ReagentPack as the EndoPack-MVL system, but also includes one normal human dermal microvascular endothelial cell product, proliferating or cryopreserved (T-25 flask or cryoamp $\geq 500,000$ cells per amp). All cells are said to be performance tested and test negative for HIV-1, hepatitis B, mycoplasma, bacteria, yeast and fungi.

Accurate Chemical has an **alternative to fetal bovine serum (FBS)** (*Reader Service No. 112*). Called Viable, the new colostrum-based medium supplement is said to offer



Viable medium — an FBS alternative.

several advantages over FBS, namely low bovine protein content, minimized batch-to-batch variation, a low endotoxin content, no FMD, BSE, IBR, BVD harmful viruses, low viscosity and a low fat content.

With the new **in-line sampling system** from Eppendorf, filtrates can be withdrawn directly from bioreactors/fermenters *in situ* (*Reader Service No. 113*). The sampling probe, ESIP 5441, is installed into the bioreactor/fermenter using standard INGOLD ports (19 mm or 25 mm). The filter



Sterile in-line sampling for fermenters and bioreactors — see Eppendorf.

membrane/sterile barrier is in direct contact with the contents of the bioreactor after its installation. This set-up enables contamination-free withdrawal of sterile-filtrated substrates at the outer connection of the sampling probe.

■ Staining

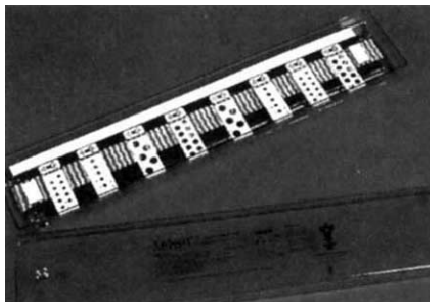
Shandon has upgraded its **tissue embedding system** to provide more efficient embedding of histological specimens, to simplify operation and to improve throughput (*Reader Service No. 114*). Called the



Histocentre 2 tissue embedding system.

Histocentre 2 and available through Life Sciences International, the system features electrically heated wax forceps in a choice of three sizes, colour-coded to ease identification. The heated forceps are designed to simplify specimen orientation by preventing the liquid wax from solidifying on the tips. A long, shallow cassette opening area provides a storage area for holding tissue prior to blocking out and a 5.2 litre wax storage facility enables users of the Histocentre 2 to work for long periods without having to refill the wax. Other features of the design include a multi-position magnifier for sample orientation purposes, a wax dispenser nozzle and touch-button control of the temperature of the hot plate. The Histocentre 2 can be used in conjunction with Shandon's Hypercenter XP enclosed tissue processor.

The magnetic **immunostaining tray** (MIST) from The Binding Site is designed to simplify the immunostaining of cells with enzyme or fluorochrome conjugates (*Reader Service No. 115*). Up to 12 glass microscope slides can be placed in the MIST and are secured by means of a magnetic strip which



Immunostaining tray for up to 12 slides.

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PRODUCT REVIEW

holds the slides in place during all incubation and washing stages. Constructed of rigid perspex with a removable lid, the MIST is designed to function as a humidity chamber for all incubation steps. When the lid is removed, the device can be tilted and the slides washed without the need to remove or handle the slides.

■ Labware

New from Carl Zeiss — the EM 906 **zoom transmission electron microscope** (*Reader Service No. 116*). The optimized electron optical hardware and computer architecture



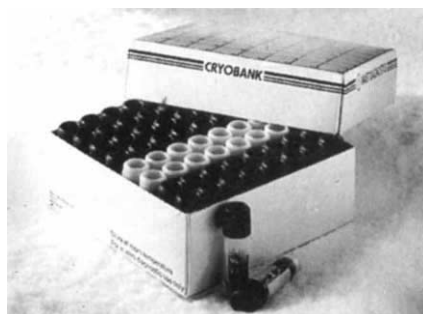
Carl Zeiss's zoom TEM.

is said to allow continuous control of all imaging parameters in real-time, giving continuous magnification zoom from $\times 40$ to $\times 600,000$ with no rotation of the image, constant image brightness regardless of

the magnification selected, continuous defraction camera length zoom from 180 mm to 1,600 mm and continuous rotation of the image through the whole magnification range. The company says that only four controls are needed in normal operation: magnification, focus, stage position (motorized) and photo record. Instrument data and parameters, including operating conditions, information about the specimen, photo conditions, comments and specimen positions may be stored on disk.

Labcaire Systems has introduced a new range of **laminar airflow cabinets** (*Reader Service No. 117*). The range includes both Class I and II microbiological safety cabinets and the Labcaire TC Cabinet designed for tissue culture and other low-risk applications. Horizontal and vertical laminar flow cabinets are also available for non-microbiological applications.

The Mast Cryobank is a **storage/retrieval system for the preservation of organisms** at low temperatures (*Reader Service No. 118*). Available from Mast Diagnostics, the system consists of chemically treated beads



Mast Diagnostics' low-temperature storage system for organisms.

covered with a cryogenic preserving solution. Each Mast Cryobank vial is said to be capable of growing 25 identical cultures, reducing storage space and processing time. In addition, the Cryobank is available in a range of colours, permitting differentiation of groups of organisms. Each pack contains 64 Cryobank tubes which are designed to fit standard freezer racks. To prevent thawing of beads during culture, Mast Cryoblock is also available, allowing the culture of one bead, while protecting the remaining beads.

PBI International has on the market a new **disinfection unit** that is designed to inactivate airborne bacteria using ultraviolet (UV) radiation (λ 254 nm) (*Reader Service No. 119*). Called the Germ-Reduc, the system works with forced ventilation: air is aspirated from the room through a special filter, flows around the UV lamps and then is expelled back into the room. The device has a stated efficiency of 92 per cent and operates with an air-flow rate of $128 \text{ m}^3 \text{ h}^{-1}$. The company also offers a hand-held air and surface sampling device, the SAS-Super90.

Brand says that its new **Transferpette pipetting device** has been designed with comfort in mind (*Reader Service No. 120*). For instance, to ensure fatigue-free pipetting, Brand has arranged all the operating elements in such a way that the operator can control every function using the thumb. To reduce operating errors, none of the controls has more than one function.

■ Shakers

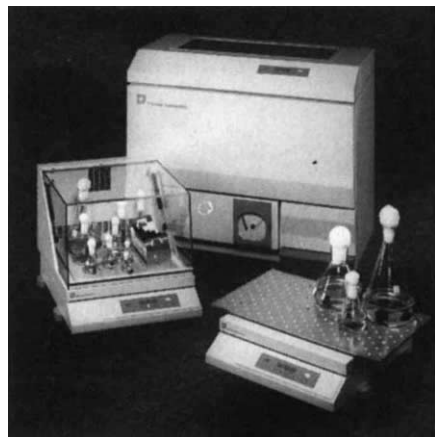
Janke & Kunkel (IKA-Labortechnik) offers the MS1 **minishaker** for the mixing of samples in test tubes, blood sampling tubes and



Multipurpose minishaker.

cuvettes (*Reader Service No. 121*). The holding plate can be changed to accommodate standard microtitre plates.

Forma has introduced a complete line of **orbital shakers** (*Reader Service No. 122*). All five tabletop and console models include microprocessor control with user-defined programmes and an independent platform



Tabletop and console orbital shakers.

monitoring system. The console models and tabletop incubator shaker feature independent overtemperature monitoring. The console models offer a HEPA-filtered airflow system and a stainless steel interior with coved corners for ease of cleaning. □

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